ATTENUATED SALMONELLA SPI2 MUTANTS AS ANTIGEN CARRIERS

Description

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Background of the invention

10 In 1996, over 17 million people world-wide, mainly in developing countries, were killed by various infections. The appearance and spread of antibiotic resistances coupled with the increase in world-wide travel has led to an increasing risk for the outbreak of pandemic infections. This possibility must be taken very seriously since, for some pathogenic bacteria, the therapeutic
15 alternatives available have been reduced to a single option. Intriguingly, pathogenic bacteria have also been discovered to be a relevant factor in many chronic diseases. Stomach cancer, for example, is the second most common cancer world-wide and is directly linked with chronic *Helicobacter pylori* infections. *Chlamydia pneumoniae* has been detected in
20 arteriosclerotic plaques and recently this bacterium has been found in the diseased regions of the brain of people suffering from Alzheimer's disease. Many autoimmune diseases, such as rheumatoid arthritis, seem to have bacterial origin. *Borrelia burgdorferi* is, in addition to many other bacteria, a prominent example of an organism causing disease affecting increasing
25 numbers of people. Finally, *Nanobacteria* have been identified in the chronically diseased kidneys of patients with crystalline deposits. Other serious chronic diseases are caused by viral pathogens, the most clinically relevant are Hepatitis B and C viruses (liver cancer) and the human papilloma virus (cervical cancer).

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The increasing clinical importance of bacterial pathogens has provoked increased discussion regarding the paradigm of medicinal treatment or

pr vention as the means to handle chronic diseases. Consistently, some chronic diseases have been successfully cured by antibiotic treatment. However, as indicated above, all micro-organisms are genetically capable of rapidly generating progenies with adequate antibiotic resistances, thus 5 impeding efficient routine treatment. Conclusively, vaccines represent an excellent alternative to pharmacological drugs, and, considering the financial aspect that disease prevention is less cost-intensive than therapy, the option of vaccination is even more attractive. Therefore, the therapeutic vaccination approach has become particularly relevant, especially with 10 respect to the treatment of cancer and chronic bacterial or viral diseases.

The most frequently practised approach uses oral delivery of either inactivated pathogens (dead vaccine) or parenteral injections of a defined mixture of purified components (subunit vaccines). Most of the dead 15 vaccines are efficacious, however, the risk that the inactivation procedure was incomplete and that the vaccinee may become infected remains a problem. Furthermore, dead vaccines very often do not cover all genetic variants that appear in nature. The subunit vaccines abolish most of the disadvantages of the traditional dead vaccines. However, they require 20 technologically advanced antigen and adjuvant preparations, which makes such vaccines relatively expensive. Furthermore, the subunit vaccines are preferentially inoculated by the parenteral route, which is not the optimal route for eliciting a broad immune response. In particular, the mucosal branch of the immune system, which is the primary line of protection 25 against many pathogens, is strongly neglected by parenteral immunisations.

Another generation of vaccines is represented by live attenuated vaccines, which are based on pathogenic bacteria or viruses that have been mutated to apathogenic variants. These variants multiply *in vivo* for a limited period 30 of time before they are completely cleared by the host. Their limited prevalence in the host tissue is sufficient to adequately provoke the host immune system, which is then able to establish a protective immune

response. From the safety aspect, live attenuated bacterial vaccines are more favoured than live attenuated viral vaccines. Should a live bacterial vaccine becomes threating for a vaccinee, the attenuated bacteria can generally be controlled by antibiotic treatment. In contrast, live viral vaccines, which use the replication apparatus of the host cell, are almost impossible to control. Live bacterial vaccines are typically administered orally and serve as excellent stimulators of the mucosal immune system. Moreover, live bacterial vaccines are also good stimulators of the systemically active immune system, namely the humoral and cellular branches. Due to these excellent immuno-stimulatory characteristics, live bacterial vaccine strains, such as *Salmonella*, are ideal carriers for expressing antigens from a heterologous pathogen. Such bivalent (or multivalent) vaccines mediate protection against two pathogens: the pathogen homologous to the carrier as well as the pathogen whose protective antigen(s) are expressed by the carrier. Although no bivalent bacterial vaccine expressing heterologous antigens is currently in use, potential carriers currently under investigation include *Bacille Calmette-Guerin* (BCG), *Salmonella* species, *Vibrio cholerae* and *Escherichia coli*.

In the attenuation process, mutations are preferentially targeted to genes that support the survival of the pathogen in the host. Initially, chemical mutation regimes were applied to the *Salmonella typhi* strain Ty2, resulting in what were thought to be perfectly attenuated pathogens capable of mediating protective immunity, in contrast to the dead homologue. However, subsequent large-scale clinical trails revealed that such strains were still not sufficiently efficacious in the prevention of typhoid fever. It appears that such strains were mutated in several genes, resulting in an over-attenuation, which adversely affects the immunogenic potential of the strain. Novel typhoid vaccine strains have been developed by the introduction of genetically defined mutations. Most of these mutations have been established in *S. typhimurium*. Infection with *S. typhimurium* causes typhoid fever-like symptoms in mice and murine salmonellosis is a well

accepted model for human typhoid. Such vaccine strains contain mutations in proteins causing deficiencies in the biosynthesis of aromatic amino acids (*e.g. aroA*, *aroC* and *aroD*) or purines (*e.g. purA* and *purE*), in the adenylate cyclase gene (*cya*) or the cAMP receptor protein (*crp*), or possess mutations affecting the regulation of several virulence factors (*phoP* and *phoQ*). However, although a number of attenuated mutants have been generated and characterised in the mouse model with regard to their role in virulence, relatively few of them have been evaluated as vaccine carriers in humans. The reason for this is that the mutants used are either still too virulent, causing severe side effects in the host, or are not sufficiently immunogenic, due to inadequate presentation to the immune system, which requires a critical level of persistence of the vaccine strain in the host for activation.

A recent study revealed that the inactivation of individual *Salmonella* genes causing attenuation of virulence directly influences the quality of an immune response against the vaccine carrier strain. From this finding, one can conclude that it might be possible to generate a variety of differently attenuated *Salmonella* vaccine strains, each with a unique profile and individual capabilities for eliciting an immune response. With this repertoire, it might be possible to tailor a vaccine strain according to specific immunological demands. As a logical consequence, one should also be able to develop attenuated *Salmonella* vaccine strains for either prophylactic or therapeutic purposes. However, the means by which such a representative repertoire of *Salmonella* vaccine strains is obtained and further developed into an efficacious vaccine must be determined.

In cases in which a *Salmonella* vaccine strain is used as a carrier for heterologous antigens, additional parameters must be considered. Traditionally, heterologous antigens have been expressed in the *Salmonella* cytosol. In the mouse typhoid model, it was demonstrated that, when heterologous antigens are expressed at high levels in the *Salmonella* cytosol, inclusion bodies are often formed, which negatively influence the

immunogenicity of the recombinant live vaccine strain in the vaccinated host. It was concluded that the formation of inclusion bodies might be fatal for the bacterium, further decreasing vitality and increasing attenuation, and thus lowering the immunogenicity. Indeed, specific expression systems that circumvent this secondary attenuation principle, e.g. the 2-phase regulated expression system, can improve the efficacy of the presentation of heterologous antigens to the host immune system.

It has been demonstrated that secretion of antigens by live attenuated *Salmonella* can be superior to intracellular expression of the same antigens both in eliciting protective T-cell responses (Hess et al., 1996; Hess et al., 1997b) and in eliciting elevated levels of antigen-specific antibody (Gentschev et al., 1998). Efficiencies of HlyA-directed secretion systems, however, are usually low (30% or less of total synthesized antigen) (Hess et al., 1997a; Hess et al., 1996), and the system seems to be problematical in *S.typhi* for export of heterologous antigens (Orr et al., 1999).

A similar immunological profile is induced by the two type III secretion systems, which are encoded by the *Salmonella* Pathogenicity Islands 1 and 2. These complex secretion machineries naturally deliver "effector proteins" into the cytosol of the infected host cell, supporting the survival of the pathogen within the host cell. By means of gene technology, the "effector proteins" can be converted into carrier vehicles for epitopes from heterologous antigens. Such chimeric "effector proteins" lose their virulent character but retain their secretory character. Consequently, the chimeric "effector protein" is delivered into the lumen of the host cell, where it is appropriately processed and subsequently stimulates the cytotoxic branch of the host immune system.

The most abundant protein secreted by *Salmonella* is flagellin (see, for example (Hueck et al., 1995)). In *S.typhimurium*, flagellin occurs in two allelic variants, FliC or FljB, while *S.typhi* carries only the FliC gene. Flagellin

is secreted via the flagellum-specific export (FEX) pathway (Macnab, 1996; Minamino and Macnab, 1999), which is homologous to the type III secretion pathway (Hueck, 1998). It also has been shown recently that the FEX pathway functions in secretion of non-flagella proteins in *Yersinia* 5 *enterocolitica* (Young et al., 1999). Like in type III secretion, the amino terminus of FliC directs secretion. Thus, a truncated version of 183 amino terminal amino acids of FliC (full length is 495 aa) is constitutively secreted in large amounts (Kuwajima et al., 1989). In analogy to type III secretion, the effective secretion signal in FliC may be as short as 10 to 20 amino 10 acids. The FliC or FljB secretion signals can potentially be used to secrete large quantities of a heterologous protein which can serve as an antigen in heterologous vaccination. It is likely that the amount of secreted antigen can be even further increased in regulatory mutants affecting the expression of flagella biosynthesis genes (Macnab, 1996; Schmitt et al., 1996) or by 15 using recombinant promoters to drive expression of the flagellin gene.

Secretion via the FEX pathway can allow the delivery of large amounts of antigen into the *Salmonella*-containing phagosome for early and efficient antigen processing and antigen presentation to the host immune system. 20 Especially the MHC class II dependent branch of the host immune system is strongly supported by the FEX pathway mediated antigen delivery.

The other known export machineries and surface display systems of Gram-negative bacteria can be also applied to bacterial vaccine carriers such as 25 *Salmonella*. In general, a good immune response is achieved when the antigen is presented on the *Salmonella* surface. However, as little is known about the immunological consequence of such antigen presentation systems, further experimental work is needed.

30 Additional immuno-modulatory effects can be achieved when environmentally regulated *Salmonella* promoters are used for the expression of heterologous antigens. For instance, the expression of a heterologous

gene in a *Salmonella* carrier strain under control of the *in vivo* regulated stress response *htrA* gene promoter resulted in a stronger immune response than was obtained when under control of the anaerobically inducible promoter of the *nirB* gene.

According to a first aspect, the present invention relates to an isolated nucleic acid molecule comprising a nucleic acid sequence comprising at least 50 nucleotides a) of the nucleic acid sequence of one of Figs.21A, B, b) of an allele of the nucleic acid sequence of one of Figs.21A, B or c) of a nucleic acid sequence which under stringent conditions hybridizes with the nucleic acid sequence of one of Figs. 21A, B.

Stringent hybridization conditions in the sense of the present invention are defined as those described by Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989), 1.101-1.104. According to this, hybridization under stringent conditions means that a positive hybridization signal is still observed after washing for 1 hour with 1 x SSC buffer and 0.1% SDS at 55°C, preferably at 62°C and most preferably at 68°C, in particular, for 1 hour in 0.2 x SSC buffer and 0.1% SDS at 55°C, preferably at 62°C and most preferably at 68°C.

In particular, the present invention relates to such a nucleic acid molecule which comprises the complete coding regions or parts thereof of the genes ssaD, ssaE, sseA, sseB, sscA, sseC, sseD, sseE, sscB, sseF, sseG, ssaG, ssaH, ssaE, ssaJ, ssrA and ssrB. The invention pertains also to such nucleic acids, wherein at least one coding region of said genes is functionally deleted.

In one embodiment, the nucleic acid molecule comprises an insertion cassette to facilitate the insertion of a heterologous nucleic acid molecule by transposon or phage mediated mechanism.

Furthermore, said nucleic acid molecules can comprise at least one heterologous nucleic acid molecule. In this case the heterologous nucleic acid molecule may be fused 5' or 3', inserted or deletion-inserted to the inventive nucleic acid molecule. By the term "deletion-inserted" it is understood that the insertion of the heterologous nucleic acid molecule is associated with a concurrent deletion of parts of the inventive nucleic acid molecule. Preferably, the nucleic acid molecule is inserted or deletion-inserted and in one preferred embodiment the heterologous nucleic acid molecule is flanked 5' and 3' by sequences of the nucleic acid molecule according to the invention, wherein each of said sequences has a length of at least 50 nucleotides, preferably 200-250 nucleotides.

Preferred, the heterologous nucleic acid molecule codes for a polypeptide or peptide, more preferred it codes for a bacterial or viral antigen or a homologue thereof or for a tumor antigen.

It is preferred that the nucleic acid molecule also comprises at least one gene expression cassette to allow for efficient expression of the heterologous nucleic acid molecule. Such gene expression cassette usually comprises elements such as promoters and/or enhancers which improve the expression of the heterologous nucleic molecule acids. Usually, such gene expression cassette comprises elements for the termination of transcription. The presence of transcription terminators, however, may be not preferred in cases where the heterologous nucleic acid molecule is to be transcribed together with other genes into a cistronic mRNA.

The nucleic acid molecule, one or more selective marker cassettes and one or more transactivator cassettes and optionally invertase cassettes for allowing the expression of the heterologous nucleic acid molecules in a one-phase system or a two-phase system. Furthermore, sequences may be present which code for a polypeptide or peptide-targeting domain and, thus, allow for the targeting of the expression product of the heterologous nucleic

acid molecule to a predetermined cell compartment such as cytosol, periplasma or outer membrane, or the secretion of said expression product, or which code for an immunostimulatory domain.

According to another aspect, the invention relates to a recombinant vector which comprises the nucleic acid molecule described above. Another aspect of the invention pertains to a cell comprising a modified inventive nucleic acid molecule as described above by insertion of a heterologous sequence or the recombinant vector. The cell may be a prokaryotic cell such as a gram-negative cell, e.g. a Salmonella cell, or it can be a eukaryotic cell such as a mammalian cell, e.g. a human cell, and, in particular, a macrophage.

According to a still further aspect, the present invention relates to a peptide or polypeptide comprising a peptide sequence comprising at least 20 amino acids a) of the sequence of one of Figs. 23A-Q, or b) of a sequence which is 60%, preferred 65% and more preferred 70% homologous to the sequence of one of Figs. 23A-Q. In particular, the invention relates to a polypeptide comprising the sequence a) of one of Figs. 23A-Q, or b) which is 60%, preferred 65% and more preferred 70% homologous to the sequence of one of Figs. 23A-Q.

Percent (%) homology are determined according to the following equation:

$$H = \frac{n}{L} \times 100$$

wherein H are % homology, L is the length of the basic sequence and n is the number of nucleotide or amino acid differences of a sequence to the given basic sequence.

Another aspect of the present invention relates to an antibody which is directed against an epitope which is comprised of the aforementioned peptide or polypeptide. The antibody may be polyclonal or monoclonal.

Methods for producing such an antibody are known to the person skilled in the art.

A further aspect of the present invention relates to a fusion protein comprising the polypeptide according to any one of the claims 17 and 18 having inserted or deletion-inserted or being fused C- or NH_2-terminally with at least one heterologous polypeptide. The heterologous polypeptide preferred is an antigen, more preferred a bacterial or viral antigen or a tumor antigen.

The present invention furthermore provides instructions for the development of a variety of potential live *Salmonella* vaccine strains with different attenuation levels, which subsequently serve as platforms for the development of recombinant live *Salmonella* vaccine carrier strains that express antigens from heterologous pathogens, thus serving as multivalent vaccines. Such recombinant live *Salmonella* vaccine carriers are equipped with modules comprising variable gene cassettes that regulate the expression of heterologous antigens in *Salmonella* and determine presentation of the heterologous antigens to the host immune system. By combinations of both systems, differently attenuated live *Salmonella* vaccine strains and variable gene cassettes, a variety of recombinant live vaccine carrier strains can be generated that have, due to their variable immunogenic characteristics, a broad application spectrum for both prophylactic and therapeutic use. The basic attenuation principle originates from novel mutations in the *Salmonella* Pathogenicity Island 2 (SPI2) gene locus. Additional mutations, which can be used either alone or in combination with mutations in sse or SPI-2 genes or in combination with the *aroA* mutation for optimal attenuation of live vaccine carrier strains, have been reported recently (Heithoff et al., 1999; Valentine et al., 1998). By combination of the individual mutations in the SPI-2 gene locus with each other and with other known attenuating gene mutations, such as *aroA*, etc., a broad repertoire of attenuation and immunogenicity can be

achieved. Different expression cassettes can be introduced on these platforms, allowing further modulation of the immune response directed against the heterologous antigens. Finally, a library of individual recombinant live *Salmonella* vaccine carrier strains is generated, covering a broad spectrum of immuno-stimulatory potential, from which a genuine live vaccine strain can be tailored for the optimal protection or treatment of humans and/or animals against specific pathogens or disease.

Thus, in a further aspect, the present invention is an attenuated gram-negative cell comprising the SPI2 gene locus, wherein at least one gene of the SPI2 locus is inactivated, wherein said inactivation results in an attenuation/reduction of virulence compared to the wild type of said cell.

Genes present in the *Salmonella* pathogenicity island 2 that encode for a variety of proteins involved in type III secretion and those that are required for systemic spread and survival within phagocytic cells are ideal candidates for attenuation of pathogenic *Salmonella* ssp.

Several gram-negative bacterial pathogens secrete certain virulence proteins via specialised type III secretion systems. Virulence factors enable pathogenic bacteria to colonise a niche in the host despite specific attacks of the immune system. The type III secretion systems comprise a large number of proteins required to transfer specific effector proteins into eukaryotic host cells in a contact-dependent manner, thus they have also been called contact-dependent secretory systems. Although several components of the secretion system apparatus show evolutionary and functional conservation across bacterial species, the effector proteins are less well conserved and have different functions. The *Yersinia* effectors YpkA and YopH have threonine/serine kinase and tyrosine phosphatase activities, respectively. The actions of these and other Yops inhibit bacterial phagocytosis by host cells, which is thought to enable extracellular bacterial proliferation. The *Shigella* Ipa proteins, secreted by the *mxi/spa* type III

secretion system, promote entry of this bacterium into epithelial cells. EspA, EspB and EspD, encoded by the locus of enterocyte effacement (LEE) of enteropathogenic *Escherichia coli* (EPEC) are required for translocation of proteins that cause cytoskeletal rearrangements and the formation of
5 pedestal-like structures on the host cell surface.

For the purposes of the present invention an "gram-negative cell comprising the SPI2 gene locus" is a cell having a gene locus that harbors genes required for the systemic spread and survival within phagocytic cells and,
10 thus, is a homologue or functional equivalent of the SPI2 locus from Salmonella. Preferred, the inventive attenuated gram-negative cell is an Enterobactericae cell, more preferred, a Salmonella cell, a Shigella cell or a Vibrio cell. In general, cells having a broad host range are preferred. Typical hosts are mammals, e.g. man, and birds, e.g. chicken. Salmonella cells are
15 more preferred, and particularly preferred is Salmonella serotype typhimurium Definitive Type 104 (DT 104).

Salmonella typhimurium is unusual in that it contains two type III secretion systems for virulence determinants. The first controls bacterial invasion of
20 epithelial cells, and is encoded by genes within a 40kb pathogenicity island (SPI1). The other is encoded by genes within a second 40kb pathogenicity island (SPI2) and is required for systemic growth of this pathogen within its host. The genes located on pathogenicity island SPI1 are mainly responsible for early steps of the infection process, the invasion of non-phagocytic host
25 cells by the bacterium. For most of the SPI1 genes, mutations result in a reduced invasiveness *in vitro*. However, mutants that are defective in invasion are not necessarily avirulent; studies in mice demonstrated that, while these mutations in SPI1 genes significantly reduced virulence upon delivery by the oral route, they had no influence on virulence following an
30 intraperitoneal route of infection. Taken together, these results indicate that mutations in genes within the pathogenicity island SPI1 do not abolish systemic infection and are therefore not very useful for the development of

a safe, attenuated *Salmonella* carrier strain. In comparison, virulence studies of SPI2 mutants have shown them to be attenuated by at least five orders of magnitude compared with the wild-type strain after both oral and intraperitoneal inoculation of mice.

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Many of the genes encoding components of the SPI2 secretion system are located in a 25kb segment of SPI2. SPI2 contains genes for a type III secretion apparatus (*ssa*) and a two component regulatory system (*ssr*), as well as candidate genes for a set of secreted effectors (*sse*) and their
10 specific chaperones (*ssc*). On the basis of similarities with genes present in other bacterial pathogens, the first 13 genes within the *ssaK/U* operon and *ssaJ* encode components of the secretion system apparatus. A number of additional genes, including *ssaC* (orf 11 in Shea *et al.,* 1996; *spiA* in Ochman *et al.*, 1996) and *ssrA* (orf 12 in Shea *et al.,* 1996; *spiR* in Ochman
15 *et al.*, 1996), which encode a secretion system apparatus protein and a two component regulatory protein, respectively, are found in a region approximately 8kb from *ssaJ*.

Preferably, the inventive attenuated gram-negative cell has inactivated at
20 least one gene selected from effector (sse) gene secretion apparatus (ssa) genes, chaperon (ssc) genes and regulation (ssr) genes. More preferably, the at least one inactivated gene is an sse, ssc and/or ssr gene, even more preferred is an sse and/or ssc gene.

25 As far as the sse genes are affected by the inactivation, the inactivated gene is preferably sseC, sseD, sseE or a combination thereof. As far as the ssr genes are affected by the inactivation, preferably at least ssrB is inactivated. As far as the ssc genes are affected by the inactivation, preferably at least sscB is inactivated.

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The inactivation of said gene of the SPI2 locus (or functional homologue thereof in cells other than Salmonella) is effected by a mutation which may

comprise deletion. Preferred are deletions of at least six nucleotides, and more preferred is a deletion of the partial and, in particular, the complete coding sequence for said gene. The mutation may also comprise the insertion of a heterologous nucleic acid molecule into said gene to be inactivated or a combination of deletion and insertion.

Pathogenic *Salmonella* ssp. serve a basis for the construction of a panel of different live *Salmonella* vaccine prototypes generated by gradual attenuations accomplished through the introduction of defined SPI2 gene locus mutations. Each resulting individual live *Salmonella* vaccine prototype is further transformed into a multivalent recombinant vaccine by the introduction of exchangeable DNA modules carrying (1) genetically engineered genes from heterologous pathogens and (2) adequate expression systems executing efficacious antigen presentation to the host immune system. In concert, these features elicit a specific immune response that either protects vaccinated hosts against subsequently invading *Salmonella* and/or other pathogens (prophylactic vaccination) or eliminates persistant pathogens, such as *Helicobacter pylori* (therapeutic vaccination).

Pathogenic *Salmonella* ssp. are gradually attenuated by mutations in individual virulence genes that are part of the SPI2 gene locus, e.g. an *sse* gene coding for an effector protein, such as *sseC*, *sseD* or *sseE*, or an *ssc* gene, such as *sscB*, coding for a chaperone, or an *ssr* gene, such as *ssrB*, coding for a regulator. Individual mutation of each of these genes leads to a unique individual grade of attenuation, which, in turn, effects a characteristic immune response at the mucosal, humoral and cellular levels. The individual grade of attenuation can be moderately increased by combinations of at least two gene mutations within the SPI2 gene locus or by combination with a mutation in another *Salmonella* gene known to attenuate virulence, e.g. an *aro* gene, such as *aroA*. A stronger grade of attenuation is achieved by mutation of a virulence gene that is part of a polycistronic gene cluster encoding several virulence factors, such as the

transcriptional unit comprising the *sseC, sseD, sseE* and *sscB* genes, such that the mutation exerts a polar effect, disrupting expression of the following genes. The grade of attenuation may directly depend on the number of virulence genes that are affected by the polar mutation as well
5 as their individual characteristics. Finally, the strongest attenuation is achieved when regulatory genes, such as *ssrB*, are mutated. Again, each mode of attenuation of a *Salmonella ssp.* leads to the generation of a live *Salmonella* vaccine strain that evokes an immune response at the mucosal, humoral and cellular levels that is characteristic for the type and/or
10 combination of attenuating mutations present in that strain. The panel of differently attenuated live *Salmonella* vaccine strains that is generated represents a pool of potential carrier strains from which that carrier can be selected that provokes the most efficacious immune response for either the prevention or eradiction of disease in conjunction with the heterologous
15 antigens that are expressed.

<u>Mutations leading to attenuation of the indicated *Salmonella* virulence genes</u> are preferentially introduced by recombinant DNA technology as defined deletions that either completely delete the selected virulence gene or result
20 in a truncated gene encoding an inactive virulence factor. In both cases, the mutation involves a single gene and does not affect expression of neighbouring genes (non-polar mutation). An insertional mutation in one of the indicated virulence genes is preferred when the selected gene is part of a polycistronic virulence gene cluster and all of the following virulence
25 genes are included in the attenuation process (polar mutation). Insertional mutations with non-polar effects are in general restricted to genes that are either singly transcribed or are localised at the end of a polycistronic cluster, such as *ssrB*. However, other attenuating mutations can arise spontaneously, by chemical, energy or other forms of physical mutagenesis
30 or as a result of mating or other forms of genetic exchange.

Thus, the mutation which results in the preparation of the inventive attenuated gram-negative cell may be a polar or non-polar mutation. Furthermore, the grade of attenuation may be modified by inactivating an additional gene outside of the SPI2 locus, for example, another virulence gene or a gene that is involved in the biosynthesis of a metabolite or a precursor thereof such as the aro genes, in particular, aroA, or any other suitable gene such as superoxide dismutase (SOD).

The attenuated cell according to the invention may furthermore comprise elements which facilitate the detection of said cell and/or the expression of an inserted heterologous nucleic acid molecule. An example of an element which facilitates the detection of the attenuated cell is a selective marker cassette, in particular, a selective marker cassette which is capable of conferring antibiotic resistance to the cell. In one embodiment, the selective marker cassette confers an antibotic resistance for an antibiotic which is not used for therapy in a mammal. Examples of elements which facilitate the expression of a heterologous nucleic acid molecule are a gene expression cassette which may comprise one or more promoter, enhancer, optionally transcription terminator or a combination thereof, a transactivator cassette, an invertase cassette for 1-phase or 2-phase expression of a heterologous nucleic acid. An example of an element which facilitates the insertion of a heterologous nucleic acid molecule is an insertion cassette.

In another aspect, the invention provides a carrier for the presentation of an antigen to a host, which carrier is an attenuated gram-negative cell according to any one of the claims 22 to 49, wherein said cell comprises at least one heterologous nucleic acid molecule comprising a nucleic acid sequence coding for said antigen, wherein said cell is capable of expressing said nucleid acid molecule or capable of causing the expression of said nucleic acid molecule in a target cell.

Preferably, said nucleic acid molecules comprises a nucleic acid sequence coding for a bacterial or viral antigen or for a tumor antigen. Examples of bacterial antigens are antigens from Helicobacter pylori, Chlamydia pneumoniae, Borrelia burgdorferi and Nanobacteria. Examples of viral antigens are antigens from Hepatitis virus, e.g. Hepatitis B and C, human papilloma virus and Herpes virus. The heterologous nucleic acid molecule may comprise a nucleic acid sequence which codes for at least one polypeptide or peptide-targeting domain and/or immunostimulatory domain. Thus, the expression product of said heterologous nucleic acid molecule may be targeted specifically to predetermined compartments such as periplasma, outer membrane, etc. The heterologous nucleic acid molecule may code for a fusion protein.

According to one embodiment the heterologous nucleic acid molecule is inserted into the SPI2 locus, preferred, into an sse gene and, more preferred, into sseC, sseD and/or sseE, in particular, sseC.

The insertion may be a polar insertion or an unpolar insertion. Generally, the introduction of an unpolar insertion is preferred, since it allows for the expression of the remaining genes of a polycistronic gene cluster, which can be used for the generation of carriers having different grades of attenuation.

Attenuated live *Salmonella* vaccines are used as carriers for specific antigens from heterologous pathogens, e.g. *Helicobacter*, etc., thus acting as a multivalent vaccine. The heterologous antigens are provided by a gene expression cassette (GEC) that is inserted by genetic engineering into the genome of an attenuated *Salmonella* strain. Preferentially, insertion of the gene expression cassette is targeted to one of the indicated virulence genes, thereby causing an insertional mutation as described in previous paragraph. In another application form, expression of the heterologous genes in the gene expression cassette is regulated by trans-acting factors

encoded by a trans-activator cassette (TC) or an invertase cassette performing a 2-phase variable expression mode. Preferentially, the insertion of the trans-activator cassette is targeted to a second chosen virulence gene, which is then inactivated. Alternatively, the gene expression cassette or the trans-activator cassette or the invertase cassette can be introduced into the *Salmonella* genome by transposon-mediated insertion, which has no attenuation effect.

The principles of genetic engineering are required to generate either deletion or insertional mutations in *Salmonella* virulence genes. Generally, a suicide plasmid carrying a mutated virulence gene cassette containing a selective marker cassette (SMC) either alone or in combination with a gene expression cassette or a trans-activator cassette or the invertase cassette is introduced into the receptor *Salmonella* strain by conjugation. The original virulence gene is replaced with the mutated virulence gene cassette via homologous recombination, and the suicide plasmid, unable to replicate in the *Salmonella* receptor strain, becomes rapidly depleted. Successfully recombined *Salmonella* can be selected based on properties (such as, but not limited to, antibiotic resistance) conferred by the product of the gene(s) within the selective marker cassette. The mutated virulence gene cassette comprises DNA sequences that are homologous to the genome of the receptor *Salmonella* strain where the original virulence gene is localised. In the case where the original virulence gene is to be completely deleted, only those genomic DNA sequences that border the original virulence gene (indicated as flanking regions) are included in the mutated virulence gene cassette. The general architecture of a mutated virulence gene cassette includes at each end a DNA sequence of at least 50 nucleotides, ideally 200 - 250 nucleotides, that is homologous to the genome segment where the original virulence gene is localised. These DNA sequences flank a selective marker cassette and the other cassettes, such as the gene expression cassette (GEC) or the trans-activator cassette (TC) or the invertase cassette. As indicated above, these cassettes are used to generate

insertional mutations which disrupt original gene expression. For in-frame deletions, a selective marker cassette is preferentially used.

The selective marker cassette (SMC) principally consists of a gene mediating resistance to an antibioticum which is able to inactivate the receptor *Salmonella* strain but which is actually not used in the treatment of Salmonellosis. Alternatively, another selectable marker can be used. The selective marker cassette is inserted in-frame in the targeted virulence gene and, consequently, the expression of the marker gene is under the control of the virulence gene promoter. Alternatively, the cassette is inserted within a polycistronic transcriptional unit, in which case the marker gene is under control of the promoter for this unit. In another application, the selective marker gene is under control of its own promoter; in this case a transcriptional terminator is included downstream of the gene. The selective marker is needed to indicate the successful insertion of the mutated virulence gene cassette into the genome of the receptor *Salmonella* strain. Furthermore, the antibiotic resistance marker is needed to facilitate the pre-clinical immunological assessment of the various attenuated *Salmonella* strains. In another application form, the selective marker is flanked by direct repeats, which, in the absence of selective pressure, lead to the recombinatorial excision of the selective marker cassette from the genome, leaving the short sequence of the direct repeat. Alternatively, the selective marker cassette can be completely removed by recombinant DNA technology. Firstly, the selective marker cassette is removed by adequate restriction endonuclease from the original mutated virulence gene cassette on the suicide plasmid leaving the flanking region sequences which are homologous to the Salmonella genome. The suicide plasmid is then transfered into the attenuated receptor *Salmonella* strain by conjugation where the SMC-depleted mutated virulence gene cassette replaces the SMC-carrying mutated virulence gene cassette by recombination. After removal of the selective marker, the attenuated *Salmonella* strain is free for

the application in humans. Transcriptional terminator sequences are generally included in the cassettes when polar mutations are established.

The gene expression cassette (GEC) comprises elements that allow, facilitate or improve the expression of a gene. In a functional mode the gene expression cassette additionally comprises one or more gene expression units derived from either complete genes from a heterologous source or fragments thereof, with a minimal size of an epitope. Multiple gene expression units are preferentially organised as a concatemeric structure. The genes or gene fragments are further genetically engineered, such that the resulting proteins or fusion proteins are expressed in the cytosol, in the periplasm, surface displayed or secreted. Furthermore the genes or gene fragments can be fused with DNA sequences encoding immunologically reactive protein portions, e.g. cytokines or attenuated bacterial toxins. The genes or gene fragments are either controlled in a one-phase mode from a promoter within the gene expression cassette or in a 2-phase mode or indirectly by a trans-activator cassette (TC). In the one-phase mode the promoter is preferentially a *Salmonella* promoter that is activated, i.e. induced, by environmental signals but also constitutive promoters of different strength can be used. In the 2-phase mode, the expression of the gene cassette is controlled by an invertase that derived from an invertase cassette. The invertase catalyses the inversion of a DNA segment comprising the gene cassette. The DNA segment is flanked on each end by an inverted repeat which is the specific substrate for the invertase finally causing two orientation of the gene cassette with respect to the gene expression cassette promoter. In the ON-orientation the gene cassette is correctly placed allowing transcription of the gene cassette. In OFF, the orientation of the gene cassette is incorrect and no transcription occurs. The invertase cassette comprises of an invertase that is controlled by a constitutive promoter or a *Salmonella* promoter induced or derepressed by environmental signals.

Heterologous antigens encoded within the gene expression cassette can be expressed under the control of a promoter, e.g. a tissue-specific promoter, which may be constitutive or inducible. The expression can be activated in a target cell, whereby a signal is transmitted from the target cell to the interior of the Salmonella cell, which signal induces the expression. The target cell, for example, can be a macrophage. The expression product may comprise a targeting domain or immunostimulatory domain, e.g. in the form of a fusion protein. The heterologous protein itself also may be a fusion protein. The heterologous antigens can be optionally expressed as cytosolic, periplasmic, surface displayed or secretory proteins or fusion proteins in order to achieve an efficacious immune response. The antigen encoding sequences may be fused to accessory sequences that direct the proteins to the periplasm or outer membrane of the *Salmonella* cell or into the extracellular milieu. If the heterologous polypeptides are secreted, secretion can occur using a type III secretion system. Secretion by the SPI2 type III secretion system is suitable. Proteins that are destined for the cytosolic compartment of the *Salmonella* do not need accessory sequences, in this case, naturally occurring accessory sequences must be removed from the genes encoding such antigens.

The accessory sequences for the periplasmatic compartment of *Salmonella* comprise a DNA sequence deduced from the amino-terminally localised signal peptide of a heterologous protein naturally translocated via the general secretion pathway, e.g. CtxA, etc.

The accessory sequences for the outer membrane compartment of *Salmonella* preferentially comprise DNA sequences deduced from the functionally relevant portions of a type IV secretory (autotransporter) protein, e.g. AIDA or IgA protease. The appropriate fusion protein contains an amino-terminally localised signal peptide and, at the carboxy-terminus, a ß-barrel shaped trans-membrane domain to which the foreign passenger

protein is coupled via a spacer that anchors the passenger protein to the bacterial surface.

The accessory sequences for secretion into the extracellular milieu comprise DNA sequences deduced from proteins naturally secreted by the type III secretion system. In a generally functional fusion protein, the heterologous antigen is fused in the centre of a protein naturally secreted by the type III pathway or at the carboxy-terminal end of the respective protein.

The transactivator cassettes (TC) provide activators which generally improve expression of the heterologous antigens encoded by the various gene expression cassettes. Such activators either directly (RNA polymerase) or indirectly (transcriptional activator) act on the transcription level in a highly specific order. Preferentially, the expression of such activators are controlled by *Salmonella* promoters which are induced *in vivo* by environmental signals. In another application form the synthesis of the activator within the transactivator cassette is regulated in a 2-phase mode. The invertase expressed by the invertase cassette places the activator encoding DNA fragment in two orientations with respect to the transcriptional promoter. In the ON-orientation the activator gene is in the correct transcriptional order. In the OFF-modus the activator is incorrectly orientated and no expression occurs.

In the simple system, the gene product of the transactivator cassette exerts its effect directly on the promoter present in the gene expression cassette, directly activating or de-repressing expression of the heterologous gene. In the complex system, activation of the promoter in the heterologous gene expression cassette is dependent upon two or more interacting factors, at least one of which (encoded in the transactivator cassette) may be regulated by external signals. Further complexity is found in cascade systems, in which the external signal does not directly exert its effect on the transactivator cassette, but rather through a multi-step process, or in

which the gene product of the transactivator cassette does not directly exert its effect on the heterologous gene expression cassette, but rather through a multi-step process.

5 According to still another aspect, the present invention is an attenuated gram-negative cell comprising the SPI2 gene locus, characterized by a lack of at least one SPI2 polypeptide, wherein said lack results in an attenuation/reduction of virulence compared to the wild type of said cell. Preferably, said missing SPI2 polypeptide is one or more effector
10 polypeptide, secretion apparatus polypeptide, chaperon polypeptide or regulatory polypeptide. Furthermore, said attenuated cell may be a carrier which then is characterized by the presence of at least one heterologous peptide or polypeptide having immunogenic properties.

15 A further aspect of the present invention is a pharmaceutical composition which comprises as an active agent an immunologically protective living vaccine which is an attenuated gram-negative cell or carrier according to the invention. The pharmaceutical composition will comprise additives such as pharmaceutically acceptable diluents, carriers and/or adjuvants. These
20 additives are known to the person skilled in the art. Usually, the composition will administered to a patient via a mucosa surface or via or via the parenteral route.

Further aspects of the present invention include a method for the
25 preparation of a living vaccine, which comprises providing a living gram-negative cell comprising the SPI2 locus and inactivating at least one gene of the SPI2 locus to obtain an attenuated gram-negative cell of the invention, and optionally inserting at least one heterologous nucleic acid molecule coding for an antigen to obtain a carrier according to the
30 invention. A further aspect pertains to a method for the preparation of a living vaccine composition comprising formulating an attenuated cell or a carrier according to the invention in a pharmaceutically effective amount

together with pharmaceutically acceptable diluents, carriers and/or adjuvants. A further aspect of the invention relates to a method for the detection of an attenuated cell or a carrier according to the invention, comprising providing a sample containing said cell and detecting a specific property not present in a wild type cell. Methods for detecting a specific property of the attenuated cell or carrier, which is not present in wild type, are known to the person skilled in the art. For example, if this specific property of the attenuated cell comprises a deletion of one or more parts of the SPI2 locus, then the presence of said cell can be detected by providing a pair of specific primers which are complementary to sequences flanking this deletion and amplifying a fragment of specific length using amplification methods such as PCR. Methods for detecting the presence of an inventive carrier comprise PCR amplification of an inserted fragment or a fragment spanning the insertion boundary, hybridization methods or the detection of the heterologous expression product or of a selective marker.

A further aspect of the invention is a method for establishing a library of attenuated gram-negative cells or carriers, respectively, according to the invention. The method comprises the preparation of attenuated recombinant vaccine strains, each having a different mutation in the SPI2 locus which results in a different degree of attenuation. The pathogenicity or virulence potential of said strains can then be determined using known methods such as determination of the LD50, and the strains are rated according to the different pathogenicities, i.e. a different grade of attenuation. Preferably, the method comprises also the determination of other parameters of interest such as the immunogenicity or the immuno-stimulatory response raised in a host. Methods for determining the immuno-stimulatory potential are known to the person skilled in the art and some of them are described in Example 6. Preferably, the immuno-stimulatory potential of the inventive attenuated cells or carriers is determined at humoral, cellular and/or mucosal level. In this way it is possible to establish a library of attenuated cells or carriers having a predetermined attenuation degree and predetermined

immuno-stimulatory properties. Thus, for each application, the strain having the desired properties can be selected specifically. For example, it will be usually preferred to select a strong attenuated strain for administration to patients which receive immunosuppressive drugs.

In a similar way, the invention allows for the establishment of libraries of attenuated carriers having defined pathogenicities and optionally immunogenicities. The establishment of a carrier library additionally will comprise the determination of the antigen presentation of said carrier strains to a host, whereby a panel of different carriers strains will be obtained having defined properties with respect to pathogenicity, immuno-stimulatory potential of carrier antigens and immuno-stimulatory potential of the heterologous antigen.

Another aspect of the invention is the use of the attenuated cell or carrier according to the invention for the preparation of a drug for the preventive or therapeutic treatment of an acute or chronic disease caused essentially by a bacterium or virus. For example, for the prevention or treatment of a Salmonella infection one will administer an attenuated Salmonella cell to raise the immune response of an affected patient. Similarly, a carrier according to the invention may be used for the preparation of a drug for the preventive or therapeutic treatment of a tumor.

The individual immuno-protective potential of each of the established recombinant *Salmonella* vaccine strains is determined in a mouse model using a pathogenic *Salmonella typhimurium* as the challenge strain.

- Determination of the virulence potential of the recombinant *Salmonella* vaccine strain: (1) Competitive index or LD50; (2) Systemic prevalence in blood, liver and spleen strictly excluded.
- Determination of the immuno-stimulatory potential of the carrier strain with a cytosolically expressed heterologous test antigen: (1)

Single oral immunisation and subsequent evaluation of the short- and long-term immune response: (a) analysis of the humoral immune response profile, (b) analysis of the mucosal immune response profile, (c) analysis of the cellular immune response profile; (2) Multiple oral immunisations and subsequent evaluation of the short- and long-term immune response: (a) analysis of the humoral immune response profile, (b) analysis of the mucosal immune response profile, (c) analysis of the cellular immune response profile.

- Determination of the immuno-stimulatory potential of the carrier strain for the delivery of heterologous DNA (DNA vaccination).

Preferentially, the *Salmonella* acceptor strain has a broad host range, exhibiting significant pathogenicity in both animals and humans. Ideally, this is a *Salmonella* strain that is strongly pathogenic for mice, such as *S. typhimurium*. After successful development of the recombinant *Salmonella* vaccine strain, the strain is directly applicable for use in both animals and humans. If such an ideal *Salmonella* acceptor strain is not satisfactory for the respective host, other host-specific *Salmonella* must be selected, such as *S. typhi* for humans.

Other aspects of the invention relate to the use of a nucleic acid molecule as shown in Fig. 21A or B or one of the Figs.22A-Q, optionally modified as described hereinabove or of a vector as described hereinabove for the preparation of an attenuated cell, a living vaccine or a carrier for the presentation of an antigen to a host and to the use of the Salmonella SPI2 locus for the preparation of an attenuated cell, a living vaccine or preferably a carrier for the presentation of an antigen to a host. In this context the term "Salmonella SPI2 locus" refers to any nucleic acid sequence, coding or not coding, and to the expression product of coding sequences.

A still further aspect of the present invention is the use of a virulence gene locus of a gram-negative cell for the preparation of a carrier for the presentation of an antigen to a host.

5 Another aspect of the invention relates to a method of therapeutically or prophylactically vaccinating an animal, e.g. a mammal, e.g. a human, against a chronic disease caused primarily by a infectious organism including preparation and administering a vaccine of the invention.

10 Still another aspect of the present invention is an isolated nucleic acid molecule comprising a nucleic acid of at least 100 nucleotides a) of the nucleic acid sequence of one of Figs.24A, B, b) of a nucleic acid sequence which under stringent conditions hybridizes with the nucleic acid sequence of one of Figs.24A, B.

15

In particular, said aspect relates to said nucleic acid molecule which is capable of inducing the expression of a nucleic acid sequence conding for a peptide or polypeptide operatively linked to said nucleic acid molecule.

20 The in vivo inducible promoter Pivi comprises a DNA fragment which carries sequences for an operator and a transcriptional promoter. Such in vivo inducible promoter can be identified by applying an adequate reporter gene approach. Two of such in vivo inducible promoters have been identified within the SPI2 locus which initiate expression of the ssaBCDE operon 25 (promoter A2) and the sseABsscAsseCDEsscBsseFG operon (promoter B), respectively. These promoters are induced by a regulative system comprising the ssrA and ssrB gene products. This regulative system is part of the SPI2 locus responsible for the activation of additional SPI2 locus genes. The regulative system is activated in macrophages by environmental

signal(s) via sensor protein SsrA. The SsrB protein finally binds at a defined DNA sequence which initiates transcription through the RNA polymerase.

In an application form the DNA fragment comprising operator/promoter sequences is inserted in front of an invertase gene or an activator gene or a gene expression cassette, thereby executing an in vivo inducible expression in bacteria carrying at least the ssrA and ssrB genes or the complete SPI2 locus.

Thus, in a further aspect, the invention relates to an expression system for the in vivo inducible expression of a heterologous nucleic acid in a target cell, comprising a carrier cell for said heterologous nucleic acid, wherein said carrier cell comprises (a) a polypeptide having the amino acid sequence shown in Fig.23P (ssrA) or a functional homologue thereof, (b) a polypeptide having the amino acid sequence shown in Fig.23Q (ssrB) or a functional homologue thereof, and (c) the nucleic acid molecule of one of Figs.24A, B or a functional homologue thereof, as described above.

The target cell may be any suitable cell but preferably it is a macrophage. The carrier cell preferably is a Salmonella cell. The target cell may also comprise one or more of the elements described above such as selective marker cassettes, gene expression cassettes, transactivator cassettes, invertase cassettes and/or insertion cassettes. Furthermore, it may comprise a heterologous nucleic acid, in particular, the heterologous nucleic acids may be inserted into a gene expression cassette, thus rendering the GEC functional.

A still further aspect of the invention relates to the use of a nucleic acid molecule comprising at least 100 nucleotides of the nucleic acid sequence

shown in one of Figs.24A, B or hybridizing therewith and having promoter activity, for the in vivo inducible expression of a heterologous nucleic acid molecule.

5 A further aspect of the present invention is the use of said nucleic acid molecule for the detection of in vivo inducible promoters.

Experimental Procedures

10 The strains, material, and methods used in the type III secretion system of the *Salmonella* Pathogenicity Island 2 (SPI2) work described above are as follows:

Mice

15

Female BALB/c (H-2^d) of 6-12 weeks of age were maintained under standard conditions according to institutional guidelines. This study was approved by an ethic committee for animal use in experimental research.

20 *Bacterial strains, phages and plasmids*

The bacterial strains, phages and plasmids used in this study are listed in Table 1. Unless otherwise indicated, bacteria were grown at 37°C in Luria Bertani (LB) broth or agar, supplemented with ampicillin (50 µg/ml),
25 kanamycin (50 µg/ml), or chloramphenicol (50 µg/ml) where appropriate. Eukaryotic cells were grown in RPMI 1640 supplemented with 10% of

foetal calf serum (FCS), 100 U/ml penicillin, 50 μg/ml streptomycin, $5x10^{-5}$ M 2-mercaptoethanol and 1 mM L-glutamine (GIBCO BRL; Prisley, Scotland). To achieve constitutive expression of ß-gal, the plasmid pAH97 (Holtel *et al.*, 1992) was electroporated into the carrier strains as described elsewhere (O'Callaghan and Charbit, 1990).

Table 1. Phages, plasmids and bacterial strains used in this work.

Phage, plasmid or strain	Description	Reference
Phages		
λ1	clone from a library of *S. typhimurium* genomic DNA in λ1059	Shea *et al.*, 1996
λ2	clone from a library of *S. typhimurium* genomic DNA in λ1059	Shea *et al.*, 1996
λ5	clone from a library of *S. typhimurium* genomic DNA in λ1059	Shea *et al.*, 1996
Plasmids		
pBluescriptKS+, pBluescriptSK+	Amp^r; high copy number cloning vectors	Stratagene, Heidelberg
pUC18	Amp^r; high copy number cloning vector	Gibco-BRL, Eggenstein
pT7-Blue	Amp^r; high copy number cloning vector	Novagen, Heidelberg

pCVD442	suicide vector	Donnenberg et al., 1991
pACYC184	Cm^r,Tet^r; low copy number cloning vector	Chang and Cohen, 1978
pGPL01	R6K ori, Amp^r; λ*pir*-dependent suicide vector for *luc* fusions	Gunn and Miller, 1996
pLB02	R6K ori, Amp^r; λ*pir*-dependent suicide vector for *luc* fusions	Gunn and Miller, 1996
pGP704	R6K ori, Amp^r; λ*pir*-dependent suicide vector	Miller and Mekalanos, 1988
pKAS32	Amp^r; λ*pir*-dependent suicide vector; *rpsl*^{S}	Skorupski and Taylor, 1996
pNQ705	R6K ori, Cm^r; λ*pir*-dependent suicide vector	Forsberg *et al.*, 1994
pSB315	Kan^r, Amp^r	Galán *et al.*, 1992
p1-6	Amp^r, 4.8kb *Pst*I/*Bam*HI fragment of λ1 in pT7-Blue	this work
p1-20	1.7kb *Bam*HI/*Hinc*II fragment of p1-6 in pKS+	this work
p1-21	*aphT* cassette in *Eco*RV site of p1-20	this work
p1-22	*Xba*I/*Kpn*I insert of p1-21 in pKAS32	this work
p2-2	Amp^r, 5,7kb *Bam*HI fragment of λ2 in pUC18	this work
p2-20	1.6kb *Hind*III/*Hinc*II fragment of p2-2 in *Hind*III/*Sma*I-digested pKS+	this work
p2-21	*aphT* cassette in *Hinc*II site of p2-20	this work
p2-22	insert of p2-21 in pKAS32	this work
p2-50	3.7kb *Bam*HI/*Kpn*I fragment of p2-2 in pKS+	this work
p5-2	Amp^r; 5.7kb *Eco*RI fragment of λ5 in pKS+	this work
p5-30	3.0kb *Pst*I/*Eco*RI fragment of p5-2 in pUC18	this work
p5-31	*aphT* cassette in *Eco*RV site of p5-30	this work

p5-33	SphI/*Eco*RI insert of p5-31 in pGP704	this work
p5-4	Amp^r; 5.8kb *Hin*dIII fragment of λ5 in pSK+	this work
p5-40	4.5kb SstI/*Hin*dIII fragment of p5-2 in pKS+	this work
p5-41	aphT cassette in *Sma*I site of p5-40	this work
p5-43	*Kpn*I/*Sst*I insert of p5-41 in pNQ705	this work
p5-5	Amp^r; *Pst*I digestion of p5-4 and religation of the larger fragment	this work
p5-50	2.6kb *Bam*HI/*Cla*I fragment of p5-2 in pKS+	this work
p5-51	*aphT* cassette in *Hin*dIII site of p5-50 after Klenow fill-in	this work
p5-53	*Xba*I/*Sal*I insert of p5-51 in pGP704	this work
p5-60	*Cla*I-digestion of p5-2 and religation of larger fragment	this work
p5-8	Amp^r, 2.2kb *Pst*I/*Hin*dIII fragment of p5-2 in pSK+	this work
p*sseA*	Cm^r; *sseA* in pACYC184	this study
p*sseB*	Cm^r; *sseB* in pACYC184	this study
p*sseC*	Cm^r; *sseC* in pACYC184	this work
E. coli strains		
DH5α	see reference	Gibco-BRL
S17-1 λ*pir*	λ*pir* phage lysogen (see reference)	Miller and Mekalanos, 1988
CC118 λ*pir*	λ*pir* phage lysogen (see reference)	Herrero *et al.*, 1990
XL1-Blue	see reference	Stratagene

S. typhimurium strains		
NCTC12023	wild-type	Colindale, UK
CS015	*phoP*-102::Tn*10d*-Cm	Miller *et al.*, 1989
CS022	*phoP*^{c}	Miller *et al.*, 1989
P2D6	*ssaV*::mTn*5*	Shea *et al.*, 1996
P3F4	*ssrA*::mTn*5*	Shea *et al.*, 1996
P4H2	*hilA*::mTn*5*	Monack *et al.*, 1996
P6E11	*spaRS*::mTn*5*	Shea *et al.*, 1996
P8G12	*ssrB*::mTn*5*	Shea *et al.*, 1996
P9B6	*ssaV*::mTn*5*	Shea *et al.*, 1996
P9B7	*ssaT*::mTn*5*	Shea *et al.*, 1996
P11D10	*ssaJ*::mTn*5*	Shea *et al.*, 1996
NP*ssaV*	*ssaV*::*aphT*, Km^{r}; non-polar mutation	Deiwick *et al.*, 1998
HH100	*sseA*Δ:::*aphT*, Km^{r}; non-polar mutation	this study
HH101	HH100 containing p*sseA*	this study
HH102	*sseB*Δ:::*aphT*, Km^{r}; non-polar mutation	this study
HH103	HH102 containing p*sseB*	this study
HH107	*sseF*Δ:::*aphT*, Km^{r}; non-polar mutation	this study
HH108	*sseG*::*aphT*, Km^{r}; non-polar mutation	this study
MvP102	Δ*sseEsscB*,_Km^{r}; non-polar mutation	this work
MvP103	*sseC*::*aphT*, Km^{r}; non-polar mutation	this work
MvP103[p*sseC*]	MvP103 containing p*sseC*	this work
MvP131	*ssaB*::*luc* in *S. typhimurium* NCTC12023	this work
MvP127	*sseA*::*luc* in *S. typhimurium* NCTC12023	this work
MvP239	*sipC*::*lacZY*, EE638 in *S. typhimurium* NCTC12023	Hueck *et al.*, 1995; this work
MvP244	*ssaB*::*luc* in *S. typhimurium* P8G12	this work

MvP266	*ssaH::luc* in *S. typhimurium* NCTC12023	this work
MvP284	*ssrA::aphT*, Km^r; non-polar mutation	this work
MvP320	*ssrB::aphT*, Km^r; non-polar mutation	this work
MvP337	in-frame deletion in *sseC*	this work
MvP338	in-frame deletion in *sseD*	this work
MvP339	in-frame deletion in *sscB*	this work
MvP340	in-frame deletion in *ssrA*	this work
SL7207	*S. typhimurium* 2337-65 *his*G46, DEL407 *aroA*::Tn*1*(Tc-s)	gift from B.A.D. Stocker
III-57 sseC	Δ sseC	this work

Example 1: Distribution of the pathogenicity island SPI-2 within different *Salmonella* strains

The presence of open reading frames of the SPI-2 region in various Salmonella isolates and E. coli K-12 was analyzed by Southern hybridization as shown in Table 2.

Table 2: Prevalence of SPI-2 genes in various Salmonella ssp. deduced from representative gene probes

Species	subspec.	serovar/serotype	*ssrAB*	*ORF*
S. enterica	I	typhimurium	+	+
S. enterica	I	typhi	+	+
S. enterica	II		+	+
S. enterica	IIIa		+	+
S. enterica	IIIb		+	+
S. enterica	IV		+	+
S. enterica	VI		+	+
S. enterica	VII		+	+
S. bongori		66:z41:--	-	+
S. bongori		44:z48:--	-	+
E. coli K-12			-	-

Presence or absence of hybridizing bands is indicated by + or -, respectively.

Hybridization

Genomic DNA of various *Salmonella* strains and *E. coli* K-12 was prepared as previously described (Hensel *et al.*, 1997a). For Southern hybridization analysis, genomic DNA was digested with *Eco*RI or *Eco*RV, fractionated on 0.6 % agarose gels and transferred to Hybond N$^+$ membranes (Amersham, Braunschweig). Various probes corresponding to the SPI-2 region were obtained as restriction fragments of the subcloned insert of λ1. Probes corresponding to ORF 242 and ORF 319 were generated by PCR using primer sets D89 (5'-TTTTTACGTGAAGCGGGGTG-3') and D90 (5'-GGCATTAGCGGATGTCTGACTG-3'), and D91 (5'-CACCAGGAACCATTTTCTCTGG-3') and D92 (5'-CAGCGATGACGATATTCGACAAG-3'), respectively. PCR was performed according to the specifications of the manufacturer (Perkin-Elmer, Weiterstadt). PCR products were submitted to agarose gel electrophoresis and fragments of the expected size were recovered and purified. Hybridization probes were labeled using the DIG labeling system as described by the manufacturer (Boehringer, Mannheim).

Example 2: Characterization of *sse* genes and construction of *sseC::aphT*, *sseD::aphT* and *sseE*Δ mutant *S. typhimurium* strains MvP103, MvP101 and MvP102

Organization of sse *and* ssc *genes*

In order to characterize SPI2 genetically and functionally, a central region of the pathogenicity island (Fig. 1A) has been cloned and sequenced. DNA fragments covering the region between *ssaC* and *ssaJ* were subcloned in plasmids p5-2 and p5-4 as indicated in Fig. 1C. The arrangement and designation of genes in the 8kb region between *ssaC* and *ssaK* is shown in Fig. 1B. This sequence will be available from the EMBL database under

accession number AJ224892 in the near future. The sequenced region extends the open reading frame (ORF) of a gene encoding a putative subunit of the type III secretion apparatus referred to as *spiB* (Ochman *et al.*, 1996). For consistency with the universal nomenclature for type III secretion system subunits (Bogdanove *et al.*, 1996) and the nomenclature of other SPI2 genes (Hensel *et al.*, 1997b), this gene has been designated *ssa*D. The deduced amino acid sequence of *ssaD* is 24% identical to YscD of *Y. enterocolitica.* This is followed by an ORF with coding capacity for a 9.3 kDa protein, 34% identical to YscE of *Y. enterocolitica*. Therefore, this gene is designated *ssaE*. A sequence of 263 bp separates *ssaE* and a set of nine genes, several of which encode proteins with sequence similarity to secreted effector proteins or their chaperones from other pathogens. These genes are separated by short intergenic regions or have overlapping reading frames and it is likely that some are co-transcribed and translationally coupled. Therefore, the genes with similarity to those encoding chaperones were designated *sscA* and *sscB*, and the others *sseA-E*. The amino acid sequence deduced from *sscA* shows 26% identity/49% similarity over 158 amino acid residues to SycD, the product of *lcrH* of *Y. pseudotuberculosis* which acts as a secretion-specific chaperone for YopB and YopD (Wattiau *et al.*, 1994). The amino acid sequence deduced from *sscB* shows 23% identity/36% similarity over 98 amino acid residues to IppI of *Shigella flexneri*. IppI is a chaperone for *S. flexneri* invasion proteins (Ipas) (Baundry *et al.*, 1988). As is the case for the secretion chaperones SycD, IppI and SicA (Kaniga *et al.*, 1995), SscB has an acidic pI (Table 3), whereas SscA has an unusually high pI of 8.8. SseB is 25% identical/47% similar to EspA of EPEC over the entire length of the 192 amino acid residue protein (Fig. 2b). SseD is 27% identical/51% similar to EspB of EPEC over 166 amino acid residues. SseC has sequence similarity to a class of effector proteins involved in the translocation of other effectors into the target host cell. These include YopB of *Y. enterocolitica*, EspD of EPEC and PepB of *Pseudomonas aerugunosa*. SseC is approximately 24% identical/48% similar to both EspD of EPEC and YopB of *Y. enterocolitica* (Fig. 2a). EspD

and YopB have two hydrophobic domains that are predicted to insert into target cell membranes (Pallen *et al.*, 1997). SseC contains three hydrophobic regions that could represent membrane-spanning domains. Other features of these predicted effector proteins are shown in Table 1.
5 Using the TMpredict program (Hofmann and Stoffel, 1993), transmembrane helices are predicted for all the effector proteins apart from SseA which is very hydrophilic. Alignments of SseC to homologs in other pathogens are shown in Fig. 2b. Conserved amino acids are mainly clustered in the central, more hydrophobic portion of the protein, but unlike YopB, there is
10 no significant similarity to the RTX family of toxins. The conserved residues in SseD are present mainly in the N-terminal half of the protein. Comparison of the deduced amino acid sequences of *sseABCDEF* with entries in the PROSITE database did not reveal the presence of any characteristic protein motifs. We subjected the predicted amino acid sequences of the *sse* genes
15 to searches using the programs COIL and MULTICOIL as described by Pallen *et al.* (1997). SseA and SseD are predicted to have one trimeric coil each, and SseC is predicted to have two trimeric coils (Table 3). Since EspB and EspD are predicted to have one and two trimeric coils, respectively (Pallen *et al.*, 1997), this provides further evidence that these proteins are
20 functionally related.

Table 3. Features of predicted proteins.

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Protein	M_r (kDa)	pI	Tm predictions	Predicted coils
SseA	12.5	9.3	hydrophilic	at least one (trimer)
SseB	21.5	4.7	one transmembrane helix	none
SseC	52.8	6.3	three transmembrane helices	at least two (trimers)
SseD	20.6	4.8	three transmembrane helices	at least one (trimer)
SseE	16.3	9.7	one transmembrane helix	none
SscA	18.1	8.8	hydrophilic	none
SscB	16.4	4.7	hydrophilic	none

30

Expression of SPI2 genes

Generation of antibodies against recombinant SPI2 proteins

5 In order to monitor the expression of the SPI2 genes *sseB, sscA* and *ssaP*, a Western blot analysis of total bacterial cells with polyclonal antibodies raised against recombinant SPI2 proteins SseB, SscA, and SsaP was performed.

10 Protein gel electrophoresis and Western blotting were performed as described elsewhere (Laemmli, 1970 and Sambrook *et al.*, 1989). Plasmids for the expression of recombinant SPI2 protein were constructed by cloning the individual SPI2 genes in plasmids pQE30, pQE31 or pQE32 (Qiagen, Hilden) in order to generate in-frame fusion to the N-terminal 6His tag. 15 Recombinant SPI2 genes were expressed in *E. coli* M15 [pREP] (Qiagen) and purified by metal chelating chromotography according to recommendations of the manufacturer (Qiagen). For immunisation, about 1 mg of recombinant SPI2 proteins were emulsified with complete and incomplete Freund's adjuvant for primary and booster immunizations, respectively. Rabbits were 20 immunized subcutaneously according to standard protocols (Harlow and Lane, 1988). SPI2 proteins were detected with antisera raised against recombinant SPI2 proteins after electrophoretical separation of proteins from total cells and transfered onto a nitrocellulose membrane (Schleicher and Schuell) using a 'Semi-Dry' blotting device (Bio-Rad) according to the 25 manufacturers manual. Bound antibody was visualized using a secondary antibody-alkaline phosphatase conjugate according to standard protocols (Harlow and Lane).

Generation of reporter gene fusions:

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Fusions of the reporter gene firefly luciferase (*luc*) to various genes in SPI2 were obtained using the suicide vectors pLB02 and pGPL01 (Gunn and

Miller, 1996), which were kindly provided by Drs. Gunn and Miller (Seattle).

For the generation of a fusion to *ssaB*, a 831 bp *Eco*RV fragment of p2-2
5 was subcloned in *Eco*RV digested pSK$^+$. For the generation of a transcriptional fusion to *sseA*, a 1060 bp *Sma*I/*Hin*cII fragment of p5-4 was subcloned in pSK$^+$. The inserts of the resulting constructs were recovered as a *Eco*RI/*Kpn*I fragment and ligated with *Eco*RI/*Kpn*I digested reporter vectors pGPL01 and pLB02. For the generation of a transcriptional fusion
10 to *ssaJ*, a 3kb *Sma*I/*Kpn*I fragment of p5-2 was directly subcloned in pGPL01 and pLB02.

Constructs with transcriptional fusions of SPI2 genes to *luc* were than integrated into the chromosome of *S. typhimurium* by mating between *E.*
15 *coli* S17-1 *λpir* harbouring the respective construct and a spontaneous mutant of *S. typhimurium* resistant to 100 μg x ml^{-1} nalidixic acid and selection for exconjugants resistant to carbenicillin and nalidixic acid. The targeted integration in SPI2 (for constructs using pGLP01) or the *zch* region (for constructs using pLB02) was confirmed by Southernanalysis. Fusions
20 were then moved into a mouse-passaged strain of *S. typhimurium* NCTC12023 by P22 transduction according to standard procedures (Maloy *et al.*, 1996).

Assay of reporter genes

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ß-galactosidase activities of reporter gene fusions were determined according to standard procedures (Miller, 1992).
Bacterial strains harbouring firefly luciferase fusions to SPI2 genes (strain MvP127, *sseA*::*luc*, strain MvP131, *ssaB*::*luc*, strain MvP266, *ssaH*::*luc*)
30 were grown in medium with various Mg^{2+} concentrations. The luciferase activity of aliquots of the cultures was determined using the Promega (Heidelberg) luciferase assay kit or custom made reagents accordingly.

Briefly, bacteria were pelleted by centrifugation for 5 min. at 20000 x g at 4°C and resuspended in lysis buffer (100 mM $KHPO_4$, pH 7.8, 2 mM EDTA, 1 % Triton X-100, 5 mg x ml^{-1} bovine serum albumin, 1 mM DTT, 5 mg x ml^{-1} lysozyme). Lysates were incubated for 15 min at room temperature with repeated agitation and subjected to a freeze/thaw cycle. Aliquots of the lysates (25µl) were transferred to microtiter plates (MicroFLUOR, Dynatech) and immediately assayed after addition of 50 µl luciferase reagent (20 mM Tricine-HCl, pH 7.8, 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 100µM EDTA, 33.3 mM DTT, 270 µM_Li_3-coenzyme A, 470 µM D(-)-luciferin, 530 µM Mg-ATP) for photon emission using the TriLux MicroBeta luminometer (Wallac, Turku). All assays were done in triplicates and repeated on independent occasions.

Expression of SPI2 genes such as ssaB *and* ssaH *is induced by low Mg^{2+} concentrations of the growth medium*

S. typhimurium wild-type strain and strains harbouring luc reporter-gene fusions to *ssaB* (strain MvP131) and to *ssaH* (strain MvP266) were grown to mid-log phase (OD at 600 nm of about 0.5) in minimal media containing high amounts of Mg^{2+} (10 mM $MgCl_2$). This medium is referred to as medium G. Bacteria were recovered by centrifugation, washed three times in minimal medium containing 8 µM Mg^{2+}. This medium is referred to as medium F. Bacteria were resuspended in medium F or medium G and growth at 37°C was continued. Aliquots of the cultures of strains MvP131 and MvP266 were withdrawn at the several different time points indicated and subjected to analysis of luciferase activity. Aliquots of the wild-type strain were withdrawn at the same time points. Protein from total bacterial cells was separated by SDS-PAGE and transferred to nicrocellulose membranes. These blots were incubated with antibodies raised against recombinant SsaP and SscA protein in order to detect proteins synthesized after the magnesium concentration shift in the magnesium concentration.

After shifting bacteria from a growth medium with high amounts of Mg^{2+} to a medium with limiting amounts of Mg^{2+}, the expression of SPI2 genes was highly induced. This induction can be monitored by using the reporter gene luc fused into different positions of SPI2. Furthermore, proteins synthesized after induction of SPI2 were detected by Western Blots. However, even in the presence of high amounts of Mg^{2+}, a low level of expression of SPI2 genes was observed.

Expression of SPI2 genes such as sseA *and* ssaB *is modulated by PhoP/PhoQ regulation*

No expression of *sseB* or *sscA* was observed during growth in various rich media, or cell culture media with or without serum. However, low amount of SsaP were detected after growth in LB or other rich media such as brain hart infusion (BHI). Growth in minimal medium containing less than 30 μM Mg^{2+} induces the expression of SPI2 genes. Such effect of the Mg^{2+} concentration has so far only been observed for PhoP/PhoQ-regulated genes. This observation is in contrast to a previous report by Valdivia and Falkow (1997) who postulated that SPI2 gene expression is independent of PhoP/PhoQ. However, in a $PhoP^c$ (constitutive) strain background (CS022, Miller *et al.*, 1989) expression of SPI2 genes was not constitutive but still dependent on the Mg^{2+} concentration of the medium. This indicates that SPI2 gene expression is modulated by PhoP/PhoQ, but that further regulatory elements such as SsrA/B are needed.

DNA cloning and sequencing

DNA preparations and genetic manipulations were carried out according to standard protocols (Sambrook *et al*, 1989). Plasmid DNA transformation of bacterial cells was performed by electroporation (O'Callaghan and Charbit, 1990).

Clones harbouring fragments of SPI2 were identified from a library of genomic DNA of *S. typhimurium* in λ 1059 which has been described previously (Shea *et al.*, 1996). The *sse* and *ssc* genes were subcloned from clone λ5 on a 5.7kb *Eco*RI fragment (p5-2) and a 5.8kb *Hin*dIII fragment (p5-4) in pBluescriptKS+ as indicated in Fig. 1 and Table 1.
DNA sequencing was performed using a primer-walking strategy. The dideoxy method (Sanger *et al.*, 1977) was applied using the Pharmacia T7 sequencing system for manual sequencing and the dye terminator chemistry for automatic analysis on a ABI377 sequencing instrument. Assembly of contigs from DNA sequences was performed by means of AssemblyLign and MacVector software (Oxford Molecular, Oxford). For further sequence analyses, programs of the GCG package version 8 (Devereux *et al.*, 1984) were used on the HGMP network.

Construction of non-polar mutations

The construction of non-polar mutations in *sseC* (MvP103), *sseD* (MvP101) and *sseE* (MvP102) are described below. All chromosomal modifications were confirmed by PCR and Southern hybridization analysis (Southern, 1975, J. Mol. Biol. 98: 503-517).

- Mutant MvP103, *sseC*. A 2.6kb fragment was recovered after *Bam*HI and *Cla*I digestion of p5-2 and subcloned in *Bam*HI/*Cla*I-digested pBluescript II KS+. The resulting construct termed p5-50 was digested by *Hin*dIII, blunt ended using the Klenow fragment of DNA polymerase and ligated to the *aphT* cassette. A 900 bp *Hin*cII fragment of pSB315 containing an aminoglycoside 3'-phosphotransferase gene (*aphT*) from which the transcriptional terminator had been removed (Galán *et al.*, 1992) was ligated in the

same orientation into the blunted-ended *Hin*dIII site of plasmid p5-50. After transformation of *E. coli* XL-1 Blue and selection for resistance against kanamycin and carbenicillin (50 μg/ml each) one clone has been chosen and the harbouring plasmid isolated. This plasmid was termed p5-51 and its identity confirmed by restriction analysis. It was further digested with *Sal*I and *Xba*I and the insert of 3.5kb was ligated to *Sal*I/*Xba*I-digested pGP704. This plasmid was electroporated into *E. coli* CC118 *λpir* and the transformants selected for resistance to kanamycin and carbenicillin (50 μg/ml each). As done before, one clone was chosen, its plasmid with the according DNA fragment in pGP704, termed p5-53, isolated and confirmed by restriction analysis. Plasmid p5-53 was electroporated into *E. coli* S17-1 *λpir* and transferred into *S. typhimurium* NCTC12023 (resistant to nalidixic acid, 100 μg/ml) by conjugation as has been described previously (de Lorenzo and Timmis, 1994). Exconjugants in which the *sseC* gene had been replaced by the cloned gene disrupted by insertion of the *aphT* cassette were selected by resistance to kanamycin and nalidixic acid (100 μg/ml). The resulting exconjugants were finally tested for a lactose-negative phenotype and their sensitivity to carbenicillin. Selected clones were further examined by Southernblot analysis. In order to exclude possible mutations which have been acquired during the cloning procedure the mutated *sseC* allele was transferred into a fresh *Salmonella* background by P22 transduction (described by Maloy *et al.*, 1996). The resulting *Salmonella* strain MvP103 was examined for the presence of the resistance cassette within the *sseC* gene by the use of PCR. Amplification was performed by using the primers E25 (5'-GAAATCCCGCAGAAATG-3') and E28 (5'-AAGGCGATAATATAAAC-3'). The resulting fragment had a size of 1.6kb for *S. typhimurium* wild-type and 2.5kb for strain MvP103.

For complementation of non-polar mutations in *sseC*, the corresponding genes were amplified by PCR from genomic DNA using a series of primers corresponding to the region 5′ of the putative start codons and to the 3′ ends of the genes. These primers introduced *Bam*HI restriction sites at the termini of the amplified genes. After digestion with *Bam*HI, the genes were ligated to *Bam*HI-digested pACYC184 (Chang and Cohen, 1978) and transferred into *E. coli* DH5*α*. The orientation of the insert was determined by PCR, and in addition, DNA sequencing was performed to confirm the orientation and the correct DNA sequence of the inserts. Plasmids with inserts in the same transcriptional orientation as the Tet^r gene of pACYC184 were selected for complementation studies and electroporated into the *S. typhimurium* strains harbouring corresponding non-polar mutations.

- Mutant MvP101, *sseD.* A 3.0kb fragment was recovered after *Pst*I and *Eco*RI digestion of p5-2 and subcloned in *Pst*I/*Eco*RI-digested pUC18 The resulting construct termed p5-30 was digested by *Eco*RV and treated with alkaline phosphatase. The *aphT* cassette was isolated as described above and ligated to the linearized plasmid p5-30 in the same orientation in the unique *Eco*RV site. After transformation of *E. coli* XL-1 Blue and selection against kanamycin and carbenicillin (50 μg/ml each) one clone has been chosen and the harbouring plasmid isolated. This plasmid was termed p5-31 and its identity confirmed by restriction analysis. p5-31 was further digested with *Sph*I and *Eco*RI, a 4.0kb fragment isolated and ligated to *Sph*I/*Eco*RI-digested pGP704. This plasmid was electroporated into *E. coli* CC118 *λpir* and transformants selected to kanamycin and carbenicillin (50 μg/ml each). As done before, one clone was chosen, its plasmid with the according DNA fragment in pGP704, termed p5-33, isolated and confirmed by restriction analysis. Plasmid p5-33 was electroporated into E. coli S17-1 *λpir* and transferred into *S.*

typhimurium NCTC12023 (resistant to nalidixic acid) by conjugation as has been described previously (de Lorenzo and Timmis, 1994). Exconjugants in which the *sseD* gene had been replaced by the cloned gene disrupted by insertion of the *aphT* cassette were selected by resistance to kanamycin and nalidixic acid (100 μg/ml). The resulting exconjugants were finally tested for a lactose-negative phenotype and their sensitivity to carbenicillin. Selected clones were further examined by Southernblot analysis. In order to exclude possible mutations which might have been accumulated during the cloning procedure the mutated *sseD* allele was transferred into a fresh *Salmonella* background by P22 transduction (described by Maloy *et al.*, 1996). The resulting *Salmonella* strain MvP101 was examined for the presence of the resistance cassette within the *sseD* gene by the use of PCR. Amplification was performed by using the primers E6 (5'-AGAGATGTATTAGATAC-3') and E28 (5'-AAGGCGATAATATAAAC-3'). The resulting fragment had a size of 0.8kb for *S. typhimurium* wild-type was used and 1.7kb in the case of strain MvP101.

\- Mutant MvP102, deletion of parts of *sseE* and *sscB.* A 4.5kb fragment was recovered after *Sst*I and *Hin*dIII digestion of p5-2 and subcloned in *Sst*I/*Hin*dIII-digested pKS+. The resulting construct termed p5-40 was digested by *Sma*I, digested with alkaline phospatase and ligated to the *aphT* cassette in the same orientation into the unique *Sma*I site created in the *sseE/sseB* deletion plasmid p5-40 as described above. After transformation of *E. coli* XL-1 Blue and selection against kanamycin and carbenicillin (50 μg/ml each) one clone was chosen and the harbouring plasmid isolated. This plasmid was termed p5-41 and its identity confirmed via restriction analysis. It was further digested with *Kpn*I and *Sst*I and the insert was ligated

to *Kpn*I/*Sst*I-digested pNQ705. This plasmid was electroporated into *E. coli* CC118 *λpir* and transformed bacteria selected to kanamycin and chloramphenicol (50 μg/ml each). As done before, one clone was chosen, its plasmid with the according DNA fragment in pNQ705,
5 termed p5-43, isolated and confirmed by restriction analysis. The resulting plasmid was used to transfer the mutated gene onto the *Salmonella* chromosome as described above. Resulting clones have been further examined by Southernblot analysis. To exclude possible mutations which might have been acquired during the cloning
10 procedure the mutated *sseE/sscB* allele was transferred into a fresh *Salmonella* background by P22 transduction (described by Maloy *et al.*, 1996). The resulting *Salmonella* strain MvP102 was examined for the presence of the resistance cassette within the *sseE/sseB* gene by the use of PCR. Amplification was performed by using the primers E6
15 (5'-AGAGATGTATTAGATAC-3') and E4 (5'-GCAATAAGAGTATCAAC-3'). The resulting fragment had a size of 1.6kb for *S. typhimurium* wild-type and a size of 1.9kb for strain MvP102.

20 Construction of mutant strains carrying in-frame deletions in sseC, sseD and sscB:

Based on the observation that a non-polar in *sseE* did not result in a significant attenuation of virulence in the mouse model (Hensel *et al.*,
25 1998), the generation of a deletion mutant for the *sseE* gene is not of interest for the generation of carrier strains.

Construction of an in-frame deletion in sse*C, mutant MvP337*

A deletion of 158 bp between codon 264 and 422 of *sseC* was generated. Plasmid p5-2 was digested by *Cla*I and the larger fragment containing the vector portion was recovered and self-ligated to generate p5-60. Plasmid p5-60 was linearized by digestion with *Hin*dIII, which cuts once within the
5 *sseC* gene. Primers *sseC*-del-1 (5'- GCT AAG CTT CGG CTC AAA TTG TTT GGA AAA C -3') and *sseE*-del-2 (5'- GCT AAG CTT AGA GAT GTA TTA GAT ACC -3') were designed to introduce *Hin*dIII sites. PCR was performed using linearized p5-60 as template DNA. The TaqPlus polymerase (Stratagene) was used according to the instructions of the manufacturer.
10 Reactions of 100 μl volume were set up using 10 μl of 10 x TaqPlus Precision buffer containing magnesium chloride, 0.8 μl of 100 mM dNTPs, 250 ng DNA template (linearized p5-8), 250 ng of each primer and 5 U of TaqPlus DNA polymerase. PCR was carried out for 35 cycles of: 95°C for 1 minute, 60°C for 1 minute, 72°C for 6 minutes. Then a final step of
15 72°C for 10 minutes was added. 10 μl of the PCR reaction were analyzed. A product of the expected size was recovered, digested by *Hin*dIII, self-ligated, and the ligation mixture was used to transform *E. coli* DH5α to resistance to carbenicillin. Plasmids were isolated from transformants and the integrity of the insert and the deletion was analyzed by restriction
20 digestion and DNA sequencing. The insert of a confirmed construct was isolated after digestion with *Xba*I and *Kpn*I and ligated to *Xba*I/*Kpn*I-digested vector pKAS32. The resulting construct was used to transform *E. coli* S17-1 λpir to resistance to carbenicillin, and conjugational transfer of the plasmid to *S. typhimurium* (Nal^R, Strep^R) was performed according to
25 standard procedures (de Lorenzo and Timmis, 1994). Exconjugants that had integrated the suicide plasmid by homologous recombination were selected by resistance to nalidixic acid and carbenicillin, and screened for sensitivity to streptomycin. Such clones were grown in LB to OD600 of about 0.5 and aliquots were plated on LB containing 250 μg/ml streptomycin to select for
30 colonies which had lost the integrated plasmid and undergone allelic

exchange. Clones resistant to streptomycin but sensitive to carbenicillin were used for further analysis. Screening of mutants with a deletion within the *sseC* locus was performed by PCR using primers *sseC*-For (5'- ATT GGA TCC GCA AGC GTC CAG AA -3') and *sseC*-Rev (5- TAT GGA TCC TCA GAT TAA GCG CG-3'). Amplification of DNA from clones containing the wild-type *sseC* allele resulted in a PCR product of 1520 bp, use of DNA from clones harbouring a *sseC* allele with an internal deletion resulted in a PCR product of 1050 bp. The integrity of clones harbouring the *sseC* deletion was further confirmed by Southern analysis of the sseC locus. Finally, the *sseC* locus containing the internal in-frame deletion was moved into a fresh strain background of *S. typhimurium* by P22 transduction (Maloy *et al.*, 1996) and the resulting strain was designated MvP 337.

Construction of an in-frame deletion in sse*D, mutant strain MvP338*

A deletion of 116 bp between codon 26 and 142 of *sseD* was generated. Plasmid p5-2 was digested by *Hin*dIII/*Pst*I and a fragment of 2.1kb was isolated and subcloned in *Hin*dIII/*Pst*I-digested vector pBluescript SK+. The resulting construct was designated p5-8. p5-8 was linearized by digestion with *Eco*RV, which cuts twice within the *sseD* gene. Primers *sseD*-del-1 (5'-ATA GAA TTC GGA GGG AGA TGG AGT GGA AG -3') and *sseD*-del-2 (5'-ATA GAA TTC GAA GAT AAA GCG ATT GCC GAC -3') were designed to introduce *Eco*RI sites. PCR was performed using linearized p5-8 as template DNA. The TaqPlus polymerase (Stratagene) was used according to the instructions of the manufacturer. Reactions of 100 µl volume were set up using 10 µl of 10 x TaqPlus Precision buffer containing magnesium chloride, 0.8 µl of 100 mM dNTPs, 250 ng DNA template (linearized p5-8), 250 ng of each primer and 5 U of TaqPlus DNA polymerase. PCR was carried out for 35 cycles of: 95°C for 1 minute, 60°C for 1 minute, 72°C for 5 minutes. Then a final step of 72°C for 10 minutes was added. 10 µl of the

PCR reaction were analyzed. A product of the expected size was recovered, digested by *Eco*RI, self-ligated, and the ligation mixture was used to transform *E. coli* DH5α to resistance to carbenicillin. Plasmids were isolated from transformants and the integrity of the insert and the deletion was analyzed by restriction mapping and DNA sequencing. The insert of a confirmed construct was isolated after digestion with *Xba*I and *Kpn*I and ligated to *Xba*I/*Kpn*I-digested vector pKAS32. The resulting construct was used to transform *E. coli* S17-1 *λpir* to resistance to carbenicillin, and conjugational transfer of the plasmid to *S. typhimurium* (Nal^R, $Strep^R$) was performed according to standard procedures (de Lorenzo and Timmis, 1994). Exconjugants that had integrated the suicide plasmid by homologous recombination were selected by resistance to nalidixic acid and carbenicillin, and screened for sensitivity to streptomycin. Such clones were grown in LB to OD600 of about 0.5 and aliquots were plated on LB containing 250 μg/ml streptomycin to select for colonies which had lost the integrated plasmid and undergone allelic exchange. Clones resistant to streptomycin but sensitive to carbenicillin were used for further analysis. Screening of mutants with a deletion within the *sseD* locus was performed by PCR using primers *sseD*-For (5'- GAA GGA TCC ACT CCA TCT CCC TC -3') and *sseD*-Rev (5- GAA GGA TCC ATT TGC TCT ATT TCT TGC-3'). Amplification of DNA from clones containing the wild-type *sseD* allele resulted in a PCR product of 560 bp, use of DNA from clones harbouring a *sseD* allele with an internal deletion resulted in a PCR product of 220 bp. The integrity of clones harbouring the *sseD* deletion was further confirmed by Southernanalysis of the *sseD* locus. Finally, the *sseD* locus containing the internal in-frame deletion was moved into a fresh strain background of *S. typhimurium* by P22 transduction (Maloy *et al.*, 1996) and the resulting strain was designated MvP338.

Construction of an in-frame deletion in sscB, *mutant strain MvP339*

A deletion of 128 bp between codon 32 and 160 of *ssc*B was generated. A 3kb *Bgl*II fragment of plasmid p5-2 was ligated into the *Bam*HI site of pBluescript KS+ to generate plasmid p5-70. Plasmid p5-70 was linearized by digestion with *Nco*I, which cuts once within the *sscB* gene. Primers *sscB*-del-1 (5'- ATG GGA TCC GAG ATT CGC CAG AAT GCG CAA -3') and *sscB*-del-2 (5'- ATG GGA TCC ACT GGC ATA AAC GGT TTC CGG -3') were designed to introduce *Bam*HI sites. PCR was performed using linearized p5-70 as template DNA. The TaqPlus polymerase (Stratagene) was used according to the instructions of the manufacturer. Reactions of 100 μl volume were set up using 10 μl of 10 x TaqPlus Precision buffer containing magnesium chloride, 0.8 μl of 100 mM dNTPs, 250 ng DNA template (linearized p5-70), 250 ng of each primer and 5 U of TaqPlus DNA polymerase. PCR was carried out for 35 cycles of: 95°C for 1 minute, 60°C for 1 minute, 72°C for 6 minutes. Then a final step of 72°C for 10 minutes was added. 10 μl of the PCR reaction were analyzed. A product of the expected size was recovered, digested by *Bam*HI, self-ligated, and the ligation mixture was used to transform *E. coli* DH5α to resistance to carbenicillin. Plasmids were isolated from transformants and the integrity of the insert and the deletion was analyzed by restriction analysis and DNA sequencing. The insert of a confirmed construct was isolated after digestion with *Xba*I and *Kpn*I and ligated to *Xba*I/*Kpn*I-digested vector pKAS32. The resulting construct was used to transform *E. coli* S17-1 λ*pir* to resistance to carbenicillin, and conjugational transfer of the plasmid to *S. typhimurium* (Nal^R, Strep^R) was performed according to standard procedures (de Lorenzo and Timmis, 1994). Exconjugants that had integrated the suicide plasmid by homologous recombination were selected by resistance to nalidixic acid and carbenicillin, and screened for sensitivity to streptomycin. Such clones were grown in LB to OD600 of about 0.5 and aliquots were plated on LB containing 250 μg/ml streptomycin to select for colonies which had lost the

integrated plasmid and undergone allelic exchange. Clones resistant to streptomycin but sensitive to carbenicillin were used for further analysis. Screening of mutants with a deletion within the *sseC* locus was performed by PCR using primers *sscB*-For (5'- ATT GGA TCC TGA CGT AAA TCA TTA TCA -3') and *sscB*-Rev (5- ATT GGA TCC TTA AGC AAT AAG TGA ATC -3'). Amplification of DNA from clones containing the wild-type *sscB* allele resulted in a PCR product of 480 bp, use of DNA from clones harbouring a *sscB* allele with an internal deletion resulted in a PCR product of 100 bp. The integrity of clones harbouring the *sseC* deletion was further confirmed by Southernanalysis of the *sscB* locus. Finally, the *sscB* locus containing the internal in-frame deletion was moved into a fresh strain background of *S. typhimurium* by P22 transduction (Maloy *et al.*, 1996) and the resulting strain was designated MvP339.

Construction of a deletion mutation in the *sseC* gene

In a further approach the complete sequence of the chromosomal *sseC* gene was deleted by allelic replacement with a deleted copy of the gene. The deletion was constructed in a suicide plasmid (pCVD442 (Donnenberg et al., 1991). First, two DNA fragments flanking the *sseC* gene (fragment A, carrying artificial *SalI* and *XbaI* sites at its 5' and 3' ends, respectively; and fragment B, carrying artificial *XbaI* and *SacI* sites at its 5' and 3' ends, respectively) were amplified by PCR. The oligonucleotides used for PCR were: 1.) sseDelfor1 GCTGTCGACTTGTAGTGAGTGAGCAAG (3' nucleotide corresponds to bp 941 in included sequence: Fig 21A); 2.) sseCDelrev2 GGATCTAGATTTTAGCTCCTGTCAGAAAG (3' nucleotide corresponds to bp 2585 in included sequence, oligo binds to reverse strand); 3.) sseCDelfor2 GGATCTAGATCTGAGGATAAAAATATGG (3' nucleotide corresponds to bp 4078 in included sequence); 4.) sseDelrev1 GCTGAGCTCTGCCGCTGACGGAATATG (3' nucleotide corresponds to bp

5592 in included sequence, oligo binds to reverse strand). The resulting PCR fragments were fused together via the *XbaI* site. The resulting fragment was cut with *SalI* and *SacI* and cloned into pCVD442 cut with *SalI* and *SacI*. The resulting plasmid was introduced into *S.typhimurium* NCTC12023 by conjugation and chromosomal integrants of the plasmid into the *sseC* locus were selected for by the plasmid-encoded ampicillin resistance marker. In a second step, clones which had lost the plasmid were screened for by loss of ampicillin resistance. The resulting clones were tested for chromosomal deletion of the *sseC* gene by PCR, and deletion of a 1455 bp fragment, comprising the entire *sseC* open reading frame, was confirmed. This Δ*sseC* mutant strain was named III-57Δ*sseC*.

Construction of a *sseC-aroA* double mutant

In order to construct a double mutant which can serve as a prototype for a live attenuated vaccine, the *sseC:aphT* (Km^r) marker from MvP103 was transferred by P22 phage transduction into *S.typhimurium* SL7207 (*his*G46 DEL407 [*aroA*544:Tn10], Tc^R) a strain carrying a stable deletion in the *aroA* gene.

Example 3: Invasion and intracellular growth in tissue culture

Intramacrophage replication of mutant strains

Several strains which are defective in their ability to replicate inside macrophages and macrophage-like cell lines have been tested, as macrophage survival and replication are thought to represent an important aspect of *Salmonella* pathogenesis *in vivo* (Fields *et al.*, 1986). It has been reported previously that a number of SPI2 mutant strains were not defective for survival or replication within RAW macrophages (Hensel *et al.*, 1997b)

but subsequent experiments have revealed that some SPI2 mutants can be shown to have a replication defect if aerated stationary phase bacterial cultures opsonized with normal mouse serum are used (see also accompanying paper: Cirillo *et al.*, 1998). The increase in cfu for different strains in RAW macrophages over a 16 h period is shown in Fig. 3. Replication defects were observed for strains carrying mutations in *ssa*V (encoding a component of the secretion apparatus), *sseB* and *sseC* and to a lesser extent for strains carrying mutations in *sseE*. Partial complementation of this defect was achieved with strains harbouring plasmids carrying functional copies of *sseB* and *sseC,* HH103 and MvP103[p*sseC*], respectively. The ability of SPI2 mutant strains to replicate inside the J774.1 macrophage cell line (Fig. 4A) and in periodate-elicited peritoneal macrophages from C3H/HeN mice (Fig. 4B) has also been tested. Similar replication defects of *S. typhimurium* carrying transposon or non-polar mutations in SPI2 genes were observed, regardless of the phagocyte cell-type examined, although the peritoneal elicited cells had superior antimicrobial activity compared to either cell line.

Macrophage survival assays

RAW 264.7 cells (ECACC 91062702), a murine macrophage-like cell line, were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% $_{foetal}$ calf serum (FCS) and 2 mM glutamine at 37°C in 5% CO_2. *S. typhimurium* strains were grown in LB to stationary phase and diluted to an OD_{600} of 0.1 and opsonized for 20 min in DMEM containing 10% normal mouse serum. Bacteria were then centrifuged onto macrophages seeded in 24 well tissue culture plates at a multiplicity of infection of approximately 1:10 and incubated for 30 min. Following infection, the macrophages were washed twice with PBS to remove extracellular bacteria and incubated for

90 min (2h post-infection) or 16 h in medium containing gentamicin (12 μg/ml). Infected macrophages were washed twice with PBS and lysed with 1% Triton X-100 for 10 min and appropriate aliquots and dilutions were plated onto LB agar to enumerate cfu.

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Survival of opsonized *S. typhimurium* strains in J774.1 cells (Ralph *et al.*, 1975) or C3H/HeN murine peritoneal exudate cells (from Charles River Laboratories, Wilmington, MA) was determined essentially as described by DeGroote *et al.* (1997), but without the addition of interferon-γ. Briefly,
10 peritoneal cells harvested in PBS with heat-inactivated 10% $_{foetal}$ calf serum 4 days after intraperitoneal injection of 5 mM sodium periodate (Sigma, St. Louis, MO) were plated in 96-well flat-bottomed microtiter plates (Becton-Dickinson, Franklin Lakes, NJ) and allowed to adhere for 2 h. Non-adherent cells were flushed out with prewarmed medium containing 10% heat-
15 inactivated $_{foetal}$ calf serum. In previous studies, we have established that >95% of the cells remaining after this procedure are macrophages. *S. typhimurium* from aerated overnight cultures was opsonized with normal mouse serum and centrifuged onto adherent cells at an effector to target ratio of 1:10. The bacteria were allowed to internalize for 15 min, and
20 washed with medium containing 6 μg/ml gentamicin to kill extracellular bacteria. At 0 h and 20 h, cells were lysed with PBS containing 0.5% deoxycholate (Sigma, St. Louis, MO), with plating of serial dilutions to enumerate colony-forming units.

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Example 4: Evaluation of safety in the *S. typhimurium* mouse model of salmonellosis

Virulence tests with strains carrying non-polar mutations

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DNA sequence analysis suggested that the *sse* genes might encode effector proteins of the secretion system, but apart from a possible polar effect from a transposon insertion in *sscA* no strains carrying mutations in these genes were recovered in the original STM screen for *S. typhimurium* virulence
5 genes using mTn*5* mutagenesis (Hensel *et al.*, 1995), and their role in virulence was unclear. To address this question, strains carrying non-polar mutations in *sseC, sseD* and *sseEsscB* (Fig. 1) have been constructed and subjected to virulence tests. Table 4 shows that all mice inoculated with strains carrying mutations *sseC* and *sseD* survived a dose of 1 x 10^4 cfu,
10 three orders of magnitude greater than the LD_{50} of the wild-type strain, which is less than 10 cfu when the inoculum is administered by the i.p. route (Buchmeier *et al.*, 1993; Shea *et al*, 1996). The same strains containing a plasmid carrying the corresponding wild-type allele were also inoculated into mice at a dose of 1 x 10^4 cfu. No mice survived these
15 infections, which shows that each mutation can be complemented by the presence of a functional copy of each gene, and that each of these genes plays an important role in *Salmonella* virulence. Strains carrying non-polar mutations in *sseEsscB* caused lethal infections when approximately 1 x 10^4 cells of each strain were inoculated into mice by the i.p. route (Table 4) and
20 were analyzed in more detail by a competition assay with the wild-type strain in mixed infections (five mice/test) to determine if they were attenuated in virulence. The competitive index, defined as the output ratio of mutant to wild-type bacteria, divided by the input ratio of mutant to wild-type bacteria, shows that the *sseEsscB* mutant was not significantly
25 different to that of a fully virulent strain carrying an antibiotic resistance marker, which implies that this gene does not play a significant role in systemic *Salmonella* infection of the mouse.

Table 4. Virulence of *S. typhimurium* strains in mice.

30

Strain	Genotype	Mouse survival after inoculation[a] with bacterial strain	Mouse survival after inoculation[a] with mutant + complementing plasmid	Competitive index *in vivo*
NCTC12023	wild-type	0/5	n.d.	0.98[b]
MvP101	Δ*sseD::aphT*	5/5	n.d.	>0.01
MvP102	Δ*sseEsscB::aphT*	4/4	n.d.	0.79
MvP103	*sseC::aphT*	5/5	0/5	>0.01 (oral) >0.01 (i.p.)

[a] Mice were inoculated intraperitoneally with 1 x 10^4 cells of each strain

10 [b] Result of competition between wild-type strain NCTC12023 and a virulent mTn*5* mutant identified in the STM screen.

15

<u>Example 5</u>: Vaccination with the *sseC::aphT*, and Δ*sseD::aphT* mutant *S. typhimurium* strains MvP103 and MvP101

20 *Strains carrying non-polar mutations as live vaccine carriers*

To confirm the suitability of the MvP101 and MvP103 mutants as live vaccine carriers their level of attenuation was evaluated by determining the LD_{50} after oral inoculation in mice. Groups of 10 mice were fed with serial
25 dilutions of either MvP101, MvP103 or the wild-type parental strain NCTCNCTC12023 and dead animals were recorded within a period of 10

days postinfection. The obtained results demonstrated that both mutants are highly attenuated when given orally to BALB/c mice (LD_{50} above 10^9) when compared with the parental strain (LD_{50} = 6.9 x 10^5 CFU).

After intraperitoneal inoculuation the LD_{50} of *S. typhimurium* NCTC12023 wild-type in BALB/c is 6 bacteria, and the LD_{50} of MvP103 in BALB/c is 2.77 x 10^6 after intraperitoneal inoculation. The mutation can be complemented by p*sseC*, but no LD_{50} determination for the complemented mutant strain was performed. LD_{50} of MvP101 in BALB/c is 3.54 x 10^6 after intraperitoneal inoculation. A partial complementation by plasmid p5-K1 was possible. An intraperitoneal LD_{50} for MvP101 [p5-K1] of 8.45 x 10^2 was determined. (Description of p5-K1: a 3.2kb PstI fragment of p5-2 containing *sseC'sseDsseEsscBsseF'* was subcloned in low copy number cloning vector pWSK29).

Determination of the LD_{50}

Doses ranging from 10^5 to 10^9 CFU of either *S. typhimurium* NCTCNCTC12023 (wild-type) or the mutants MvP103 and MvP101 were orally inoculated into groups of 10 mice and survival was recorded over 10 days.

LD_{50} of S typhimurium wild-type and mutant strains MvP101 and MvP103 after intraperitoneal infection was determined by inoculation of doses ranging from 10^1 to 10^7 CFU into groups of 5 female BALB/c mice of 6-8 weeks of age. Survival was recorded over a period of three weeks. The LD_{50} dose of the challenge strains was calculated by the method of Reed and Muench (Reed and Muench, 1938).

Immunization protocols

For vaccination, bacteria were grown overnight until they reach medium log phase. Then, they were harvested by centrifugation (3,000 x *g*) and resuspended in 5% sodium bicarbonate. Mice were immunized four times at 15 day intervals by gently feeding them with the bacterial suspension 5 (10^9 CFU/mouse) in a volume of approximately 30 μl. Control mice were vaccinated with the carrier, lacking plasmid.

Cytotoxicity assay

10 Spleen cells were obtained from mice 14 days after the last immunization and $2x10^6$ effector cells were restimulated *in vitro* for 5 days in complete medium supplemented with 20 U/ml of rIL-2 and 20 μM of the ßGP1 peptide (ß-gal p876-884, TPHPARIGL), which encompasses the immunodominant H-2L^d-restricted ß-gal epitope. After restimulation, the 15 assay was performed using the [^{3}H]-thymidine incorporation method. In brief, $2x10^6$ of P815 cells per ml were labelled with [^{3}H]-thymidine for 4 h in either complete medium or complete medium supplemented with 20 μM of ßGP1 peptide and used as target cells. Following washing, $2x10^5$ labelled targets were incubated with serial dilutions of effector cells in 200 μl of 20 complete medium for 4 h at 37°C. Cells were harvested and specific lysis was determined as follows: [(retained c.p.m. in the absence of effectors) - (experimentally retained c.p.m. in the presence of effectors) /retained c.p.m. in the absence of effectors] x 100.

25 Example 6: Evaluation of the induced immune response

Induction of mucosal immune responses after oral vaccination

To achieve protection against mucosal pathogens using live *Salmonella* 30 carriers, elicitation of an efficient mucosal response is highly desirable.

Therefore, the presence of ß-gal-specific antibodies in intestinal washes from mice immunized with either MvP101, MvP103 or SL7207 carrying pAH97 was investigated 52 days after immunization. As shown in Fig. 5., immunization with all three carriers stimulate the production of significant
5 amounts of β-gal-specific IgA and, to a lesser extent, favor the transudation of antigen-specific IgG in the intestinal lumen. No statistically significant differences were observed among the mucosal responses to the different recombinant clones.

10

Cellular immune responses triggered after oral immunization with sseC and sseD mutants expressing β-gal

To evaluate the efficacy of the antigen-specific T cell responses generated
15 in immunized mice, spleen cells were enriched in CD4+ T cells and restimulated *in vitro* during four days with ß-gal. As shown in Fig. 6, although antigen-specific CD4+-enriched spleen cells were generated after vaccination with the three carriers, MvP103 and MvP101 were significantly more efficient than SL7207 (*P* 0.05) at triggering specific cellular immune
20 response. In contrast, cells isolated from mice immunized with the carrier alone failed to proliferate in the presence of ß-gal.

To investigate the Th-type of immune response triggered by immunization, the content of IFN-γ, IL-2, IL-4, IL-5, IL-6 and IL-10 was measured in the
25 supernatant fluids of restimulated cells. The results demonstrated that a predominant Th1 response pattern was induced in mice immunized with all the carriers. IFN-γ was the only cytokine with significantly increased levels in comparison to those observed in supernatants from spleen cells isolated from mice immunized with plasmidless carriers (Fig. 7). Interestingly, in
30 agreement with the IgG isotype patterns, the levels of IFN-γ detected in

supernatants from cells of mice immunized with MvP103 [pAH97] were significantly higher (*P* 0.05) than those from animals receiving either MvP101 [pAH97] or SL7207 [pAH97] (Fig. 7).

Antigen-specific antibody responses generated in mice orally immunized with the attenuated S. typhimurium vaccine carriers expressing the model antigen ß-gal

Groups of mice were immunized with the recombinant strains MvP101 [pAH97] and MvP103 [pAH97]. To estimate the efficacy of the prototypes another group was vaccinated with the well-established carrier strain SL7207 [pAH97]. The abilities of the different carriers to induce a systemic humoral response was determined by measuring the titer of ß-gal-specific antibodies in the serum of vaccinated mice. As shown in Fig. 8, significant titers of ß-gal-specific IgG and IgM antibodies were detected at day 30 in all vaccinated animals. In contrast to the IgM titers which reach a plateau at day 30, the titers of IgG steadily increased until day 52 from immunization when the experiment was concluded. Although all tested carriers exhibit an excellent performance, the MvP103 mutant was the most efficient at inducing anti-ß-gal IgG antibodies (*P* 0.05). No significant levels of ß-gal-specific IgA were detected in mice immunized with any of the three recombinant clones (data not shown).

To determine the subclass distribution of the anti-ß-gal IgG, serum samples were analyzed for specific levels of IgG1, IgG2a, IgG2b and IgG3. The results shown in Fig. 9 demonstrate that the main ß-gal-specific IgG isotype present in sera of all immunized mice was IgG2, suggesting of a predominant Th1 response. Interestingly, a lower concentration of IgG1 (*P* 0.05) was observed in mice immunized with MvP103 than in those

receiving MvP101 and SL7202, indicating a similar response pattern in animals immunized with the last two carriers.

5 *Sample collection*

Serum samples were collected at different time points and monitored for the presence of ß-gal-specific antibodies. At day 52 after immunization, intestinal lavages were obtained by flushing the small intestine with 2 ml of 10 PBS supplemented with 50 mM EDTA, 0.1% bovine serum albumin and 0.1 mg/ml of soybean trypsin inhibitor (Sigma). Then, the lavages were centrifuged (10 min at 600 x *g*) to remove debris, supernatants were removed and supplemented with phenylmethylsulfonyl fluoride (10 mM) and NaN_3, and stored at -20°C.

15

Antibody assays

Antibody titres were determined by an enzyme-linked immunosorbent assay 20 (ELISA). Briefly, 96 well Nunc-Immuno MaxiSorp™ assay plates (Nunc, Roskilde, Denmark) were coated with 50 µl/well ß-gal (5 µg/ml) in coating buffer (0.1 M Na_2HPO_4, pH 9.0). After overnight incubation at 4°C, plates were blocked with 10% FCS in PBS for 1 h at 37°C. Serial two-fold dilutions of serum in FCS-PBS were added (100 µl/well) and plates were 25 incubated for 2 h at 37°C. After four washes with PBS-0.05% Tween 20, secondary antibodies were added: biotinylated γ-chain specific goat anti-mouse IgG, µ-chain specific goat anti-mouse IgM, α-chain specific goat anti-mouse IgA antibodies (Sigma, St. Louis, MO) or, to determine IgG subclass, biotin-conjugated rat anti-mouse IgG1, IgG2a, IgG2b and IgG3 (Pharmingen) 30 and plates were further incubated for 2 h at 37°C. After four washes, 100

μl of peroxidase-conjugated streptavidin (Pharmingen, St. Diego, CA) were added to each well and plates were incubated at room temperature for 1 h. After four washes, reactions were developed using ABTS [2,2'-azino-bis-(3-ethybenzthiazoline-6-sulfonic acid)] in 0.1 M citrate-phosphate buffer (pH 4.35) containing 0.01% H_2O_2. Endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution which gave an optical density at 405 nm 0.1 unit above the values of the negative controls after a 30 min incubation.

To determine the concentration of total Ig present in the intestinal lavages, serial dilutions of the corresponding samples were incubated in microtiter plates that had been coated with goat anti-mouse IgG, IgM and IgA as capture antibodies (100 μg/well, Sigma) and serial dilutions of purified mouse IgG, IgM and IgA (Sigma) were used to generate standard curves. Detection of antigen-specific Ig was performed as described above.

Induction of antigen-specific CTL responses in mice orally immunized with the carrier strains expressing ß-gal

The elicitation of MHC class I restricted responses are particularly important for protection against many intracellular pathogens and tumors. It has been shown that antigen-specific CD8+ CTL can be generated both *in vitro* and *in vivo* after immunization with recombinant *Salmonella* spp. expressing heterologous antigens. Therefore, we considered it important to determine whether the tested carriers were also able to trigger a β-gal-specific CTL response. Spleen cells were collected from mice vaccinated with either MvP101 [pAH97], MvP103 [pAH97] or SL7207 [pAH97] at day 52 from immunization and restimulated *in vitro* with βGP1-pulsed syngenic spleen cells for 5 days. As shown in Fig. 10, the spleen cells from mice immunized

with either of the three constructs induced significant lysis of βGP1-loaded target cells compared with unloaded controls. The more efficient responses were observed using the carrier strain MvP103. The lysis was mediated by CD8+ T cells since the cytotoxic activity was completely abrogated when CD8+ T effector cells were depleted (data not shown).

Cytokine determination

Culture supernatants were collected from proliferating cells on days 2 and 4, and stored at -70°C. The determination of IL-2, IL-4, IL-5, IL-6, IL-10 and IFN-γ was performed by specific ELISA. In brief, 96-well microtiter plates were coated overnight at 4°C with purified rat anti-mouse IL-2 mAb (clone JESG-1A12), anti-IL-4 mAb (clone 11B11), anti-IL-5 mAb (clone TRFK5), anti-IL-6 mAb (clone MP5-20F3), anti-IL-10 mAb (clone JES5-2A5), and anti-IFN-γ mAb (clone R4-6A2) (Pharmingen). After three washes, plates were blocked and two-fold dilutions of supernatant fluids were added. A standard curve was generated for each cytokine using recombinant murine IL-2 (rIL-2), rIL-4, rIL-5, rIL-6, rIFN-γ, and rIL-10 (Pharmingen). Plates were further incubated at 4°C overnight. After washing, 100 μl/well of biotinylated rat anti-mouse IL-2 (clone JES6-5H4), IL-4 (clone BVD6-24G2), IL-5 (clone TRFK4), IL-6 (clone MP5-32C11), IL-10 (clone SXC-1) and INF-γ (clone XMG1.2) monoclonal antibodies were added and incubated for 45 min at RT. After six washes, streptavidin-peroxidase conjugated was added and incubated for 30 min at RT. Finally, the plates were developed using ABTS.

Depletion of CD8+ spleen cells.

The CD8+ cell subset was depleted using MiniMACS Magnetic Ly-2 Microbeads according to the manufacturer's instructions (Miltenyi Biotec). Depleted cell preparations contained 1 % CD8+ cells.

FACScan analysis

Approximately $5x10^5$ cells were incubated in staining buffer (PBS supplemented with 2% FCS and 0.1% sodium azide) with the desired antibody or combination of antibodies for 30 min at 4°C. After washes, cells were analysed on a FACScan (Becton Dickinson). The monoclonal antibodies used were FITC-conjugated anti-CD4 and anti-CD8 (clones H129.19 and 53-6.7; Pharmingen).

Example 7: Cell proliferation

Cell proliferation assay

Spleen cell suspensions were enriched for CD4+ T cells using MiniMACS Magnetic Ly-2 and indirect goat-anti-mouse-IgG Microbeads according to the instructions of the manufacturer (Mitenyi Biotec GmbH, Germany). Cell preparations contained > 65% of CD4+ cells. Cells were adjusted to $2x10^6$ cells/ml in complete medium supplemented with 20 U/ml of mouse rIL-2 (Pharmigen), seeded at 100μl/well in a flat-bottomed 96-well microtiter plate (Nunc, Roskilde, Denmark) and incubated for four days in the presence of different concentrations of soluble ß-gal. During the final 18 hours of culture 1 μCi of [^{3}H]-thymidine (Amersham International, Amersham, U.K.) was added per well. The cells were harvested on paper filters using a cell harvester and the [^{3}H]-thymidine incorporated into the DNA of proliferating cells was determined in a β-scintillation counter.

Example 8: Characterization of *ssr* genes and construction and characterization of the *ssr* mutant *S. typhimurium* strains MvP284 , MvP320 and MvP333

5

Homology of the two component regulator genes ssrA and ssrB of SPI2 with other bacterial proteins

The SPI2 gene *ssrA* encodes a protein similar to sensor components of
10 bacterial two component regulatory systems as has been described before (Ochman *et al.*, 1996). For consistency with the nomenclature of SPI2 virulence genes (Hensel *et al.*, 1997b; Valdivia and Falkow, 1997), this gene is designated *ssrA*. Downstream of *ssrA*, an ORF with coding capacity for a 24.3 kDa protein was identified. This gene shares significant similarity
15 with a family of genes encoding transcriptional activators like DegU of *Bacillus subtilis*, UvrY of *E. coli* and BvgA of *Bordetella pertussis*. Therefore, it is likely that the protein acts as the regulatory component of the *ssr* system and the gene was designated *ssrB*.

20

Inverse regulation of SPI1 and SPI2

The expression of the type III secretions systems of SPI1 and SPI2 is tightly regulated by environmental conditions. While SPI1 is induced during late
25 log/early stationary phase after growth in rich media of high osmolarity and limiting O_2 (oxygen) concentration, no induction of SPI2 gene expression was observed. In contrast, after growth in minimal medium with limiting amounts of Mg^{2+} (8 μM) the *ssaB*::*luc* fusion was highly expressed while the *sip*C::*lac*Z fusion was not expressed. The expression of the *ssaB*::*luc*
30 fusion is dependent on the function of SsrA/B, since there is no expression in the *ssrB*-negative background strain P8G12 (Hensel *et al.*, 1998). The expression of the *sipC*::*lac*Z fusion is dependent on HilA, the transcriptional

regulator of SPI1. We also observed that a mutation in *ssrB* affects expression of the *sipC*::*lac*Z fusion. This indicates that SPI2 has a regulatory effect on the expression of SPI1 genes.

5 Bacterial strains harbouring a luc fusion to *ssa*B in SPI2 (strain MvP131) and a *lac*Z fusion to *sip*C in SPI1 (strain MvP239) were grown under conditions previously shown to induce SPI gene expression. Bacteria were grown over night in minimal medium containing 8 μM Mg^{2+} or over night in LB broth containing 1 % NaCl (LB 1%NaCl). The Luc activity of strain MvP131 and 10 ß-galactosidase activity of strain MvP239 were determined. As a control, both reporter fusions were assayed in the *ssrB* negative strain background of P8G12.

Expression levels of *lacZ* reporter-gene fusions to SPI genes were assayed 15 as described by Miller, 1992.

Construction and analysis of *sseA* reporter gene fusion

20 A 1.1kb *Sma*I/*Hinc*II fragment of p5-4 was subcloned into pGPL01, a suicide vector for the generation of *luc* fusions (Gunn and Miller, 1996). The resulting construct, in which 1.0kb upstream and 112 bp of *sseA* is transcriptionally fused to *luc* was used to transform *E. coli* S17-1 *λpir*, and conjugational transfer to *S. typhimurium* performed as described previously 25 (Gunn and Miller, 1996). Strains that had integrated the reporter gene fusion into the chromosome by homologous recombination were confirmed by PCR and Southern hybridization analysis. Subsequently, the fusion was moved by P22 transduction into the wild-type and various mutant strain backgrounds with mTn*5* insertions in SPI1 or SPI2 genes (Maloy *et al.*, 30 1996). As a control, a strain was constructed harbouring a chromosomal integration of pLB02, a suicide plasmid without a promoter fusion to the *luc* gene (Gunn and Miller, 1996). For the analysis of gene expression, strains

were grown for 16 h in minimal medium with aeration. Aliquots of the bacterial cultures were lysed and luciferase activity was determined using a luciferase assay kit according to the manufacturer's protocol (Boehringer Mannheim). Photon detection was performed on a Microplate scintillation/luminescence counter (Wallac, Turku). All assay were done in triplicate, and replicated on independent occasions.

Expression of *sseA* is dependent on SsrAB

To establish if the *sse* genes are part of the SPI2 secretion system, the expression of an *sse*A::*luc* reporter gene fusion, integrated by homologous recombination into the chromosome of different SPI2 mutant strains, has been investigated (Fig. 11). Transcriptional activity of *sse*A in a wild-type background during growth in minimal medium was dramatically reduced by inactivation of the SPI2 two-component system. Transposon insertions in *ssrA* (mutant strain P3F4) and *ssrB* (mutant strain P8G12), encoding the sensor component and the transcriptional activator, respectively, resulted in 250 to 300-fold reduced expression of *sseA*. Inactivation of *hilA*, the transcriptional activator of SPI1 (Bajaj *et al.*, 1996), had no effect on *sseA* gene expression. Transposon insertions in two genes encoding components of the SPI2 type III secretion apparatus (*ssaJ*::mTn*5* and *ssaT*::mTn*5*; mutant strains P11D10 and P9B7; Shea *et al.*, 1996) also had no significant effect on the expression of *sseA*. These data show that SsrA/B is required for the expression of *sseA,* but that *hilA* is not.

Expression of SPI2 genes within macrophages is dependent on SsrA/B

The presence of *S. typhimurium* within eukaryotic cells (macrophages) induces the expression of SPI2 genes as indicated by analysis of fusions to

ssaB and *ssaH*. This expression is dependent on the two component regulatory system SsrA/B encoded by SPI2.

The murine macrophage-line cell line J744 was used for this experiment. Macrophages were infected at a multiplicity of infection of 10 bacteria per macrophage with MvP131 (*luc* fusion to *ssaB*), MvP266 (*luc* fusion of *ssaH*) and MvP244 (*luc* fusion to *ssaB* in a *ssrB* negative background). Extracellular bacteria were killed by the addition of gentamicin (20 μg/ml). At various time points, macrophages were lysed by the addition of 0.1% Triton X-100, and intracellular bacteria were enumerated by plating serial dilutions onto LB agar plates. A further aliquot of the bacteria was recovered and the luciferase activity was determined. Luciferase activities were expressed a relative light emission per bacteria.

Effects of a mutation in *ssrB* on the secreted effector protein of SPI1 SipC

Analysis of proteins secreted into the growth medium by the *S. typhimurium* SPI2 mutant strain MvP320 (non-polar mutation in *a*, Fig. 12) revealed the absence or strong reduction in the amounts of the secreted SPI1 effector protein (Hensel *et al.*, 1997b). These SPI2 mutants are also reduced in their ability to invade cultured epithelial cells or cultured macrophages (Hensel *et al.*, 1997b). To examine this phenomenon in greater detail, we expressed recombinant SipC (rSipC) and raised antibodies against rSipC in rabbits. In Western blots, antiserum against rSipC reacted with a 42 kDa protein from precipitates of culture supernatants of *S. typhimurium* wild-type strain NCTC12023. No reaction was observed with supernatants from cultures of EE638, a strain deficient in SipC (Hueck *et al.*, 1995). Furthermore, in Western blots SipC could not be detected in culture supernatants of the SPI2 mutants MvP320. However, SipC was detected in culture supernatants of other SPI2 mutants like P2D6 (*ssaV*::mTn*5*), P9B6 (*ssaV*::mTn*5*) and NP*ssaV* (*ssaV*::*aphT*) (Deiwick *et al.*,

1998). The detection by antiserum of SipC in culture supernatants of various strains was in accord with the presence or absence of SipC as detected by SDS-PAGE. Further it was analyzed whether the absence of SipC in culture supernatants of SPI2 mutant strains was due to defective
5 secretion of SipC via the type III secretion system or reduced synthesis of SipC in these strains. Antiserum against rSipC was used to detect SipC in pellets of cultures grown under inducing conditions for the expression of SPI1 genes (i.e. stationary phase, high osmolarity, low oxygen) (Bajaj *et al.*, 1996). Analysis of wild-type and strains carrying various mutations in SPI1
10 and SPI2 genes indicated highly reduced amounts of SipC in the mutants with a non-polar mutation in *ssrB*. However, SipC was detected at levels comparable to those observed in pellets of wild-type cultures and SPI2 mutant strains P2D6, P9B6 and NP*ssaV*. The effect on SipC synthesis is not due to reduced growth rates or reduced protein levels in SPI2 mutants,
15 since both parameters were comparable for the wild-type and SPI2 mutants.

Effects of a mutation in the SPI2 gene *ssrB* on the expression of SPI1 genes

20

In order to assay the effect of SPI2 mutations on the expression of SPI1 genes, previously characterized fusions of *lacZ* to various SPI1 genes (Bajaj *et al.*, 1995; Bajaj *et al.*, 1996) were transduced into the SPI2 mutant MvP320 and various SPI1 mutants to generate a set of reporter fusion
25 strains. The expression of the reporter ß-galactosidase in cultures grown under conditions inducing for SPI1 expression (see above) was assayed. A Tn insertion in *hilA* (P4H2) reduced the expression of *prgK* as well as *sipC*, while an insertion in *spaRS* (P6E11) only affected the expression of *sipC*. Some mutant strains with a mutation in the SPI2 gene *ssrB* encoding a
30 components of the two component regulatory system showed reduced expression of reporter fusions to *prgK* and *sipC* (Fig. 11). The effects on the expression of both genes was similar. Other mutant strains with Tn

insertions in *ssaV* (P2D6, P9B6), as well as mutant NP*ssaV* harbouring a non-polar insertions in *ssaV,* had levels of expression of *prgK* and *sipC* comparable to that of corresponding reporter fusions in a wild-type genetic background. Analysis of *lacZ* fusions to *prgH* and *invF* revealed a similar effect on expression as shown for *prgK* and *sipC*.

A mutation in the SPI2 gene *ssrB* affects expression of the SPI1 regulator *hilA*

Analysis of reporter fusions to *sip*C and *prg*K indicated that expression of genes in two different operons of SPI1 can be affected by SPI2 mutations, suggesting that these mutations affect other SPI1 genes involved in regulation of *sipC* and *prgK.* It has been demonstrated previously that the expression of SPI1 genes is under the control of the transcriptional activator HilA (Bajaj *et al.*, 1995; Bajaj *et al.*, 1996). The expression of *hil*A was therefore analyzed in the presence of a SPI2 mutation in *ssrB.* The SPI2 mutant strain MvP320 had largely diminished levels of *hilA* expression. Again, very low levels of *hilA* expression were observed in mutants that had reduced levels of *prgK* and *sipC* expression. To analyze whether the effect of the SPI2 mutation on *sipC* expression resulted from the reduced expression of *hilA*, we next performed complementation experiments in various mutant strains harbouring pVV135 (constitutive expression of *hilA*) (Bajaj *et al.*, 1996) or pVV214 (expression of *hilA* from the native promoter) (Bajaj *et al.*, 1995). In accordance with a previous study (Bajaj *et al.*, 1995), the *hilA* mutation of strain P4H2 was complemented by pVV214. However, the *sipC* expression was not restored in the mutant strain MvP320 harbouring either pVV135 or pVV214.

Construction of the *ssrA* and *ssrB* mutant *S.typhimurium* strains MvP284 and MvP320

- Mutant MvP284, *ssrA*. The *ssrA* gene (Fig. 12) was subcloned from the phage clone λ2 derived plasmid p2-2 on a 5.7kb *Bam*HI fragment in pUC18 as indicated in Table 1. A 1.6kb fragment was recovered after *Hin*dIII and *Eco*RV digestion of p2-2 and subcloned in *Hin*dIII/*Hin*cII-digested pBluescript II KS+. The resulting construct termed p2-20 was digested with *Hin*cII and dephosphorylated with alkaline phosphatase. The *aphT* cassette was isolated as described above and ligated to the linearized plasmid p2-20 in the same orientation into the unique *Hin*cII site. After transformation of *E. coli* XL-1 Blue and selection against kanamycin and carbenicillin (50 μg/ml each) one clone has been chosen and the harbouring plasmid isolated. This plasmid was termed p2-21 and its identity proved via restriction analysis. p2-21 was further digested with *Kpn*I and *Xba*I, a 2.5kb fragment isolated and ligated to *Kpn*I/*Xba*I-digested pKAS32. This plasmid was electroporated into *E. coli* CC118 λ*pir* and transformants selected to kanamycin and carbenicillin (50 μg/ml each). As done before, one clone was chosen, its plasmid with the according DNA fragment in pKAS32, termed p2-22, isolated and confirmed by restriction analysis. Plasmid p2-22 was electroporated into *E. coli* S17-1 λ*pir* and transferred into *S. typhimurium* NCTC12023 (streptomycin resistant) by conjugation as has been described previously (de Lorenzo and Timmis, 1994). Exconjugants in which the *ssrA* gene had been replaced by the cloned gene disrupted by insertion of the *aphT* cassette were selected by its growth on M9+glucose minimal medium agar plates (Maloy *et al.*, 1996) and its resistance to kanamycin and carbenicillin (100 μg/ml). The resulting exconjugants were finally shown to have a lactose negative phenotype and to be sensitive to kanamycin and streptomycin. Selected clones were further examined by Southernblot

analysis. In order to exclude possible mutations which might have been developed during the cloning procedure the mutated *ssrA* allele was transfered into a fresh *Salmonella* background by P22 transduction (described by Maloy *et al.*, 1996). The resulting *Salmonella* strain MvP284 was examined for the presence of the resistance cassette within the *ssrA* gene by the use of primers *ssrA*-For (5'- AAG GAA TTC AAC AGG CAA CTG GAG G-3') and *ssrA*-Rev (5- CTG CCC TCG CGA AAA TTA AGA TAA TA -3'). Amplification of DNA from clones containing the wild-type *ssrA* allele resulted in a PCR product of 2800 bp, use of DNA from clones harbouring a *ssrA* allele disrupted by the *aphT* cassette resulted in a PCR product of 3750bp. The resulting *Salmonella* strain MvP320 was examined for the presence of the resistance cassette within the *ssrB* gene by the use of Southern hybridization analysis of total DNA of exconjugants.

Mutant MvP320, *ssrB*. The *ssrB* gene (Fig. 12) was subcloned from the phage clone λ1 derived plasmid p1-6 on a 4.8kb *Pst*I/*Bam*HI-fragment in pT7-Blue as indicated in Table 1. A 1.7kb fragment was recovered after *Bam*HI and *Hinc*II digestion of p1-6 and subcloned in *Bam*HI/*Hinc*II-digested pBluescript II KS+. The resulting construct termed p1-20 was digested with *Eco*RV and dephosphorylated with alkaline phosphatase. The *aphT* cassette was isolated as described above and ligated to the linearized plasmid p1-20 in the same orientation into the unique *Eco*RV site. After transformation of *E. coli* XL-1 Blue and selection against kanamycin and carbenicillin (50 μg/ml each) one clone has been chosen and the harbouring plasmid isolated. This plasmid was termed p1-21 and its identity confirmed by restriction analysis. p1-21 was further digested with *Kpn*I and *Xba*I, a 2.5kb fragment isolated and ligated to *Kpn*I/*Xba*I-digested pKAS32. This plasmid was electroporated into *E. coli* CC118 λ*pir* and transformed bacteria selected to kanamycin and carbenicillin (50

μg/ml each) was performed. As done before, one clone was chosen, its plasmid with the according DNA fragment in pKAS32, termed p1-22, isolated and confirmed by restriction analysis. Plasmid p1-22 was electroporated into *E. coli* S17-1 *λpir* and transferred into *S. typhimurium* NCTC12023 (streptomycin resistant) by conjugation as has been described previously (de Lorenzo and Timmis, 1994). Exconjugants in which the *ssrB* gene had been replaced by the cloned gene disrupted by insertion of the *aphT* cassette were selected by its growth on M9+glucose minimal medium agar plates (Maloy *et al.*, 1996) and its resistance to kanamycin and carbenicillin (100 μg/ml). The resulting exconjugants were finally shown to have a lactose negative phenotype and to be sensitive to kanamycin and streptomycin. Selected clones were further examined by Southernblot analysis. In order to exclude possible mutations which might have been acquired during the cloning procedure the mutated *ssrB* allele has been transferred into a fresh *Salmonella* background by P22 transduction (described by Maloy *et al.*, 1996). Screening of mutants with a insertion of the *aphT* cassette within the *ssrB* locus was performed by PCR using primers *ssrB*-For (5'- CTT AAT TTT CGC GAG GG -3') and *ssrB*-Rev (5'- GGA CGC CCC TGG TTA ATA -3'). Amplification of DNA from clones containing the wild-type *ssrB* allele resulted in a PCR product of 660 bp, use of DNA from clones harbouring a *ssrB* allele disrupted by insertion of the *aphT* cassette resulted in a PCR product of 1600 bp. The resulting *Salmonella* strain MvP320 was examined for the presence of the resistance cassette within the *ssrB* gene by the use of Southern hybridization analysis of total DNA of exconjugants.

Construction of the mutant strain MvP340 carrying an in-frame deletion in *ssrA*

A deletion of 407 codons between codon 44 and 451 of *ssrB* was generated. Plasmid p2-2 was digested by *Bam*HI and *Kpn*I, a fragment of 3.7kb was recovered and subcloned in pBluescript KS+ to generate p2-50. Plasmid p2-50 was linearized by digestion with *Pst*I, which cuts once within the subcloned fragment of the *ssrA* gene. Primers *ssrA*-del-1 (5'- GGT CTG CAG GAT TTT TCA CGC ATC GCG TC -3') and *ssrB*-del-2 (5'- GGT CTG CAG AAC CAT TGA TAT ATA AGC TGC -3') were designed to introduce *Pst*I sites. PCR was performed using linearized p2-50 as template DNA. The TaqPlus polymerase (Stratagene) was used according to the instructions of the manufacturer. Reactions of 100 μl volume were set up using 10 μl of 10 x TaqPlus Precision buffer containing magnesium chloride, 0.8 μl of 100 mM dNTPs, 250 ng DNA template (linearized p2-50), 250 ng of each primer and 5 U of TaqPlus DNA polymerase. PCR was carried out for 35 cycles of: 95°C for 1 minute, 60°C for 1 minute, 72°C for 6 minutes. Then a final step of 72°C for 10 minutes was added. 10 μl of the PCR reaction were analyzed. A product of the expected size was recovered, digested by *Pst*I, self-ligated, and the ligation mixture was used to transform *E. coli* DH5α to resistance to carbenicillin. Plasmids were isolated from transformants and the integrity of the insert and the deletion was analyzed by restriction analysis and DNA sequencing. The insert of a confirmed construct was isolated after digestion with *Xba*I and *Kpn*I and ligated to *Xb*aI/*Kp*nI-digested vector pKAS32. The resulting construct was used to transform *E. coli* S17-1 *λpir* to resistance to carbenicillin, and conjugational transfer of the plasmid to *S. typhimurium* (Nal^R, $Strep^R$) was performed according to standard procedures (de Lorenzo and Timmis, 1994). Exconjugants that had integrated the suicide plasmid by homologous recombination were selected by resistance to nalidixic acid and carbenicillin, and screened for sensitivity to streptomycin. Such clones were grown in LB to OD600 of about 0.5 and aliquots were plated on LB containing 250 μg/ml streptomycin to select for

colonies which had lost the integrated plasmid and undergone allelic exchange. Clones resistant to streptomycin but sensitive to carbenicillin were used for further analysis. Screening of mutants with a deletion within the *ssrA* locus was performed by PCR using primers *ssrA*-For (5'- AAG GAA
5 TTC AAC AGG CAA CTG GAG G-3') and *ssrA*-Rev (5- CTG CCC TCG CGA AAA TTA AGA TAA TA -3'). Amplification of DNA from clones containing the wild-type *ssrA* allele resulted in a PCR product of 2800 bp, use of DNA from clones harbouring a *ssrA* allele with an internal deletion resulted in a PCR product of 1580 bp. The integrity of clones harbouring the *ssrA*
10 deletion was further confirmed by Southernanalysis of the *ssrA* locus. Finally, the *ssrA* locus containing the internal in-frame deletion was moved into a fresh strain background of *S. typhimurium* by P22 transduction (Maloy *et al.*, 1996) and the resulting strain was designated MvP340.

15 Southern hybridization

Genomic DNA of *Salmonella* was prepared as previously described (Hensel *et al.*, 1997). For Southern hybridization analysis, genomic DNA was digested with *Eco*RI or *Eco*RV, fractionated on 0.6 % agarose gels and
20 transferred to Hybond N^+ membranes (Amersham, Braunschweig). Various probes corresponding to the *ssrA* and *ssrB* region were obtained as restriction fragments of the subcloned insert of $\lambda 1$ and $\lambda 2$.

25 Example 9: Evaluation of safety of *S. typhimurium* strain MvP320

For competition assays between *S. typhimurium* wild-type and the mutant strain MvP320, bacteria were grown in LB to an optical density at 600 nm of 0.4 – 0.6. Cultures were diluted and aliquots of the two cultures were
30 mixed to form an inoculum containing equal amounts of both strains. The ratio of both strains was determined by plating dilutions on LB plates containing antibiotics selective for individual strains. An inoculum of about

10^4 colony forming units (cfu) was used to infect 6 to 8 weeks old female BALB/c mice (Charles River Breeders, Wiga) by injection into the peritoneal cavity. At several time points after infection mice were sacrificed by cervical dislocation and the bacterial load of liver and spleen was
5 determined by plating tissue homogenates using the 'WASP' (Meintrup, Lähden) spiral plating device. Plating was performed using LB plates containing 50 μg/ml kanamycin or 100 μg/ml nalidixic acid to select for the mutant strains or the wild-type, respectively.

10 Strain MvP320 harbouring the *aphT* gene cassette in *ssrB* was recovered in at least 1000-fold lower numbers than the *S. typhimurium* wild-type strain. These data indicate that *ssrB* contributes significantly to systemic infections of *S. typhimurium* in the mouse model of salmonellosis.

15

Statistical analysis of all experiments.

Statistical significance between paired samples was determined by Student's *t* test. The significance of the obtained results was determined
20 using the statgraphic plus for windows 2.0 software (Statistical Graphic Corp.).

Example 10: Characterization of the in vivo inducible P_{ssaE} Promoter (Promoter B, Fig.24B)

25

The promoter which is located upstream of *ssaE* (P_{ssaE}, formerly called Promoter B) was shown to be regulated by the *ssrAB* locus. A DNA fragment comprising nucleotide 800 to 120 (800-1205) in the included sequence (Fig.21A) was shown to confer *ssrB*-dependent regulation upon
30 the expression of a reporter gene (gfp) fused to the promoter. The DNA fragment was cloned on a low-copy plasmid in front of the gfp gene. As has been shown previously for other reporter gene constructs, induction of

expression from P_{ssaE} (800-1205) was observed in magnesium minimal medium (Deiwick et al., 1999) and was dependent on the presence of a chromosomal wild type allele of *ssrB*. A shorter DNA fragment, comprising nucleotide 923 to 1205 (923-1205) in the included sequence, did not
5 confer regulation upon expression of gfp. However, expression was reduced compared to the P_{ssaE} (800-1205) fragment and was not induced in magnesium minimal medium nor was it dependent on *ssrB*. Thus, the P_{ssaE} (800-1205) fragment comprises promoter active and regulatory sequences, probably including an SsrB-binding site.

10

D scription of the drawings

Fig. 1. Map of *Salmonella* Pathogenicity Island 2 **(A)** indicating the positions of the mutations in strains MvP101, MvP102, and MvP103 **(B)**. A partial restriction map of the genomic region is shown, and the positions of plasmid inserts relevant for this work are indicated **(C)**. B, *Bam*HI; C, ClaI; E, *Eco*RI; P, *Pst*I; V, *Eco*RV; S, *Sma*I; EMBL database accession numbers are indicated for the sequences in **(A)**.

Fig. 2a. Alignment of the deduced SseB amino acid sequence to EspA of EPEC (Elliot *et al.*, 1998). The ClustalW algorithm of the MacVector 6.0 program was used to construct the alignments. Similar amino acid residues are boxed, identical residues are boxed and shaded.

Fig. 2b. Alignment of the deduced SseC amino acid sequence to EspD of EPEC (Elliot *et al.*, 1998), YopB of *Yersinia enterocolitica (*Hakansson *et al.*, 1993*)*, and PepB of *Pseudomonas aeruinosa* (Hauser *et al.*, 1998). The ClustalW algorithm of the MacVector 6.0 program was used to construct the alignments. Positions where at least three amino acid residues are similar are boxed, where at least three residues are identical are boxed and shaded.

Fig. 3. Intracellular accumulation of *S. typhimurium* SPI2 mutants in RAW 264.7 macrophages. Following opsonization and infection, macrophages were lysed and cultured for enumeration of intracellular bacteria (gentamicin protected) at 2 h and 16 h post-infection. The values shown represent the fold increase calculated as a ratio of the intracellular bacteria between 2 h and 16 h post-infection. Infection was performed in in triplicates for each strain and the standard error from the mean is shown.

Fig. 4. Intracellular survival and replication of SPI2 mutant *S. typhimurium* in **(A)** J774.1 cells and **(B)** periodate-elicited peritoneal macrophages from C3H/HeN mice. After opsonization and internalization, phagocytes were lysed and cultured for enumeration of viable intracellular bacteria at time 0
5 h. The values shown represent the proportion of this intracellular inoculum viable at 20 h ± the standard error of the mean. Samples were processed in triplicate, and each experiment was performed at least twice.

Fig. 5. ß-gal-specific antibodies in intestinal lavages of mice orally
10 immunized with either MvP101[pAH97], MvP103 [pAH97], SL7207 [pAH97] or MvP101 at day 52 after immunization. Results are expressed as percentage of the corresponding total Ig subclass present in the intestinal lavage, the SEM is indicated by vertical lines. Significant levels of antigen-specific IgM could not be detected in any of the groups. The results
15 obtained with MvP103 and SL7207 (not shown) were similar to those for MvP101.

Fig. 6. β-gal-specific proliferative response of CD4+ enriched spleen cells from mice orally immunized with either MvP101 [pAH97], MvP103
20 [pAH97], SL7207 [pAH97] or MvP101. Cells were restimulated *in vitro* during a 4 day incubation with different concentrations of soluble β-gal. The values are expressed as mean cpm of triplicates; the SEM was in all cases lower than 10%. Background values obtained from wells without the stimulating antigen were subtracted. Results obtained with MvP103 and
25 SL7207 (not shown) were similar to those obtained with MvP101.

Fig. 7. IFN-γ present in supernatants from cultured CD4+ enriched spleen cells of mice orally immunized with either MvP101 [pAH97], MvP103 [pAH97], SL7207 [pAH97] or plasmidless MvP101 at day 2 and 4 of
30 culture. Spleen cells were isolated from mice at day 52 after immunization, and CD4+ enriched populations were restimulated *in vitro* for four days in the presence of soluble β-gal (20 μg/ml). IFN-γ production was determined

by ELISA, results represent the means of three determinations. The SEM is indicated by vertical lines, similar results were obtained using any of the plasmidless carriers (not shown). No significant differences with the control groups were observed when IL-2, IL-4, IL-5, IL-6 and IL-10 were tested (not shown).

Fig. 8. Kinetics of the β-gal-specific serum IgG (closed symbols) and IgM (open symbols) antibody responses in mice (n = 5) after oral immunization with either MvP101 [pAH97] (triangle), MvP103 [pAH97] (circle), SL7207 [pAH97] (square) or plasmidless MvP101 (diamond). Results are expressed as the reciprocal log_2 of the geometric mean end point titer (GMT), the SEM was in all cases lower than 10%. Similar results were obtained using any of the plasmidless carriers (not shown), immunizations are indicated by arrows.

Fig. 9. Subclass profiles of the β-gal-specific IgG antibodies present in the serum of mice (n = 5) orally immunized with either MvP101 [pAH97], MvP103 [pAH97], SL7207 [pAH97] or plasmidless MvP101 at day 52 post-immunization. Results are expressed as ng/ml, the SEM is indicated by vertical lines. Similar results were obtained using any of the plasmidless carriers (not shown).

Fig. 10. Recognition of the MHC class I-restricted βGP1 epitope by lymphocytes primed *in vivo* in mice by oral vaccination with either MvP101 [pAH97], MvP103 [pAH97], SL7207 [pAH97] or plasmidless MvP101. Spleen cells from immunized mice were restimulated *in vitro* five days in the presence of 20 μM βGP1. At the end of the culture, lymphocytes were tested in a $[^3H]$-thymidine-release assay using P815 (open symbols) and βGP1-loaded P815 (closed symbols) as targets. Results are mean values of triplicate wells (one out of three independent experiments is shown) and are expressed as: [(retained cpm in the absence of effectors) - (experimentally retained cpm in the presence of effectors) / retained cpm in the absence of

effectors] x 100; SEM were lower than 5% of the values. Similar results were obtained using any of the plasmidless carriers (not shown).

Fig. 11. Expression of an *sseA::luc* fusion in wild-type and mTn*5* mutant strains of *S. typhimurium*.

Fig. 12. Map of *Salmonella* Pathogenicity Island 2 **(A)** indicating the positions of the mutations in strains MvP284 and MvP320 **(B)**. A partial restriction map of the genomic region is shown, and the position of inserts of plasmids relevant for this work is indicated **(C)**. B, *Bam*HI; C, ClaI; H, *Hin*dIII; P, *Pst*I; V; S, *Sma*I; *Eco*RV; II, *Hin*cII.

Fig. 13. Model for the transcriptional organization of SPI2 virulence genes. This model is based on the observation of the transcriptional direction of SPI2 genes, characterization of promoter activities

Fig.14 shows the principle of how mutations having a different grade of attenuation can be generated. As shown in A, the inactivation of one effector gene such as sse results in a low grade of attenuation. As shown in B, the additional inactivation of a gene located outside the SPI2 locus such as aroA results in a medium grade of attenuation. By insertional mutation with a polar effect all genes in a polycistronic cluster are affected which results in a high grade of attenuation, as shown in C. As shown in D, the inactivation of a regulatory gene such as ssrB results in a supreme attenuation.

Fig.15 shows the principle of insertional mutation by example of insertional mutation into a virulence gene. Different cassettes such as SMC, GEC, TC and/or invertase cassette may be inserted into a cloned virulence gene, thus yielding an inactivated virulence gene which may be introduced into a cell by homologous recombination using a virulence gene cassette.

Fig.16 illustrates the selective marker cassette.

Fig.17 illustrates the gene expression cassette and the induction thereof in a two-phase system. The gene expression cassette comprises a promoter, optionally a gene cassette comprising one or more expression units and optionally one or more transcriptional terminators for the expression units and/or a transcribed sequence 5' to the gene expression cassette.

Fig.18 shows the structural requirements of the gene expression unit for the delivery of heterologous antigens into various compartments, i.e. accessory sequences that direct the targeting of the expression product.

Fig.19 shows a transactivator cassette in a one-phase system and a two-phase system.

Fig.20 shows different modes of gene expression as realized by the combination of different accessory sequences and/or cassettes in a one-phase system and a two-phase system.

Fig.21A shows the genomic sequence of a region of the SPI2 locus from Salmonella comprising the complete sequences of the genes ssaE to ssaI and partial sequences of ssaD and ssaJ (cf. Fig.12).

Fig.21B shows the nucleotide sequence of a region of the SPI2 locus from Salmonella comprising the coding sequences for ssrA and ssrB.

Figs.22A-Q each show the nucleotide sequence of the respective gene indicated.

Figs.23A-Q each show the amino acid sequence of the respective polypeptide indicated.

Figs.24A,B each show a nucleotide sequence comprising an in vivo inducible promoter.

SEQUENCE LISTING

SEQ ID NO: 1	genomic region	nucleic acid	FIG 21A
SEQ ID NO: 2	genomic region	nucleic acid	FIG 21B
SEQ ID NO: 3	sseA	nucleic acid	FIG 22A
SEQ ID NO: 4	sseA	translation product	FIG 23A
SEQ ID NO: 5	sseB	nucleic acid	FIG 22B
SEQ ID NO: 6	sseB	translation product	FIG 23B
SEQ ID NO: 7	sseC	nucleic acid	FIG 22C
SEQ ID NO: 8	sseC	translation product	FIG 23C
SEQ ID NO: 9	sseD	nucleic acid	FIG 22D
SEQ ID NO: 10	sseD	translation product	
SEQ ID NO: 11	sseD	protein	FIG 23D
SEQ ID NO: 12	sseE	nucleic acid	FIG 22E
SEQ ID NO: 13	sseE	translation product	FIG 23E
SEQ ID NO: 14	sseF	nucleic acid	FIG 22F
SEQ ID NO: 15	sseF	translation product	FIG 23F
SEQ ID NO: 16	sseG	nucleic acid	FIG 22G
SEQ ID NO: 17	sseG	translation product	FIG 23G
SEQ ID NO: 18	sscA	nucleic acid	FIG 22H
SEQ ID NO: 19	sscA	translation product	FIG 23H
SEQ ID NO: 20	sscB	nucleic acid	FIG 22I
SEQ ID NO: 21	sscB	translation product	FIG 23I
SEQ ID NO: 22	ssaD	nucleic acid	FIG 22J
SEQ ID NO: 23	ssaD	translation product	FIG 23J
SEQ ID NO: 24	ssaE	nucleic acid	FIG 22K
SEQ ID NO: 25	ssaE	translation product	FIG 23K
SEQ ID NO: 26	ssaG	nucleic acid	FIG 22L
SEQ ID NO: 27	ssaG	translation product	FIG 23L
SEQ ID NO: 28	ssaH	nucleic acid	FIG 22M
SEQ ID NO: 29	ssaH	translation product	FIG 23M
SEQ ID NO: 30	ssaI	nucleic acid	FIG 22N
SEQ ID NO: 31	ssaI	translation product	FIG 23N
SEQ ID NO: 32	ssaJ	nucleic acid	FIG 22O
SEQ ID NO: 33	ssaJ	translation product	FIG 23O
SEQ ID NO: 34	ssrA	nucleic acid	FIG 22P
SEQ ID NO: 35	ssrA	translation product	FIG 23P
SEQ ID NO: 36	ssrB	nucleic acid	FIG 22Q
SEQ ID NO: 37	ssrB	translation product	FIG 23Q
SEQ ID NO: 38	Promoter A2	nucleic acid	FIG 24A
SEQ ID NO: 39	Promoter B	nucleic acid	FIG 24B
SEQ ID NO: 40	Esp A	protein	FIG 2A
SEQ ID NO: 41	Esp D	protein	FIG 2B
SEQ ID NO: 42	Yop B	protein	FIG 2B
SEQ ID NO: 43	Pep B	protein	FIG 2B
SEQ ID NO: 44	D89	nucleic acid	page 33
SEQ ID NO: 45	D90	nucleic acid	page 33

SEQ ID NO: 46	D91	nucleic acid	page 33
SEQ ID NO: 47	D92	nucleic acid	page 33
SEQ ID NO: 48	E25	nucleic acid	page 41
SEQ ID NO: 49	E28	nucleic acid	page 41
SEQ ID NO: 50	E6	nucleic acid	page 42
SEQ ID NO: 51	E4	nucleic acid	page 43
SEQ ID NO: 52	sseC-del1	nucleic acid	page 44
SEQ ID NO: 53	sseE-del1	nucleic acid	page 44
SEQ ID NO: 54	sseC-For	nucleic acid	page 45
SEQ ID NO: 55	sseC-Rev	nucleic acid	page 45
SEQ ID NO: 56	sseD-del1	nucleic acid	page 45
SEQ ID NO: 57	sseD-del2	nucleic acid	page 45
SEQ ID NO: 58	sseD-For	nucleic acid	page 46
SEQ ID NO: 59	sseD-Rev	nucleic acid	page 46
SEQ ID NO: 60	sscB-del1	nucleic acid	page 47
SEQ ID NO: 61	sscB-del2	nucleic acid	page 47
SEQ ID NO: 62	sscB-For	nucleic acid	page 47
SEQ ID NO: 63	sscB-Rev	nucleic acid	page 47/48
SEQ ID NO: 64	ssrA-For	nucleic acid	page 66
SEQ ID NO: 65	ssrA-Rev	nucleic acid	page 66
SEQ ID NO: 66	ssrB-For	nucleic acid	page 67
SEQ ID NO: 67	ssrB-Rev	nucleic acid	page 67
SEQ ID NO: 68	ssrA-del1	nucleic acid	page 67
SEQ ID NO: 69	ssrB-del2	nucleic acid	page 68

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